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I. NÁSZ

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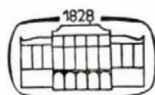
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INDEX

TOMUS XXVIII

Fasciculus 1

Körmendy, B., Bánki, M., Szabó, I.: Isolation of Mycobacteria from Contaminated Material on Selective Media	1
Horváth, I., Duncan, W. P., Bullard, J. C.: Cultivation of Pathogenic <i>Treponema pallidum</i> in vitro	7
Ezepchuk, Y. V., Morgan, S. D., Major, P.: A Simple Procedure for Isolation and Purification of A-Type Staphylococcus Enterotoxin	25
Gupta, J. K., Shirkot, C. K., Dhawan, S.: Isolation and Mutation of Cellulolytic Fungi	31
Rudnai, O., Csórián, E., Lányi, B., Ádám, M. M., Milch, H.: Salmonella and Shigella Surveillance in Hungary 1972-1976. I. Salmonella Surveillance	37
Rudnai, O., Straub, I., László, V. G., Hajnal, A., Lányi, B.: Salmonella and Shigella Surveillance in Hungary 1972-1976. II. Shigella Surveillance	53
Nguyen, T. K., Milch, H.: Klebsiella and Enterobacter Strains Derived from Hospital Infections. I. Correlation Between Species, Phage Type and Antibiotic Sensitivity	67
Antoni, F., Jobbágy, A., Puskás, M.: Surface Immunoglobulins of Tonsil Lymphocytes	83
Anderlik, P., Szeri, I., Bános, Zs., Wessely, M., Radnai, B.: Effect of Bordetella pertussis Vaccine on Neonatally Thymectomized Mice	91
Rozgonyi, F., Biacs, P., Szitha, K., Kiss, J.: Effect of Methicillin on the Fatty Acid Composition of Phospholipids in Methicillin Sensitive Staphylococcus aureus	97
Csiszár, K., Lányi, B.: Proticine Typing of Serologically Defined Proteus Strains	111
Czirók, É., Csik, M., Börzsönyi, M.: Incidence in Enteritis of Enterobacteriaceae Isolates Possessing Human Colonization Factor Antigen. New Human Colonization Factors	119
In memoriam György Ivánovics	129

Fasciculus 2

Gupta, J. K., Shirkot, C. K., Dhawan, S.: Growth of Bacteria and Yeast on Enzymically Degraded Alkali Treated Rice and Wheat Straws	131
Bakalivanov, D., Kostov, O.: Soil Microbiological Assessment of Toxicity of Alkanoate, Amide and Other Herbicides	141
Ádám, É., Nász, I.: Connections between the Hexon Polypeptides in the Two-Dimensional Hexon Crystalline Array	147
Gönczöl, É., Boldogh, I., Vácsi, L.: IgG-Fc-Binding Receptors in Cells Abortively Infected, or Transformed, by Human Cytomegalovirus	157
Kramer, M., Kecskés, É., Horváth, I.: Guanosine Polyphosphate Production of Escherichia coli Stringent and Relaxed Strains in the Stationary Phase of Growth	165
Milch, H., Nguyen T. K.: Klebsiella and Enterobacter Strains Derived from Hospital Infections. II. Occurrence and Characterization of R-, Lac- and Col-Plasmids and their Clinical-Epidemiological Significance	171

Tóth, M., Lengyel, A., Béládi, I., Nász, I.: Characterization of a Parvovirus Strain Isolated From Human Adenovirus Type 12	197
Mándi, Y., Molnár, J.: Effect of Chlorpromazine on Conjugal Plasmid Transfer and Sex Pili	205

Fasciculus 3

Lantos, J., Fekete, J., Király, K.: R-plasmid Study of an Outbreak Caused by Multiresistant Strains of <i>Salmonella panama</i>	211
Ádám, É., Nász, I.: Mutual Orientation of Adenovirus Hexon Polypeptides in a Two-Dimensional Crystalline Array	219
Šula, L.: The Colonial Microtexture of Human Mycobacterial Strains Isolated in Burma	229
Nyerges, G., Jaszovszky, I.: Reliability of the Rabbit Pyrogen Test and of the Limulus Test in Predicting the Pyrogenicity of Vaccines in Man	235
Chautard, P., Guiraud, J. P., Galzy, P.: Inulinase Activity of <i>Pichia polymorpha</i>	245
Billiau, A.: Pharmacokinetic and Pharmacological Aspects of Interferon Therapy in Man	257
Bodo, G.: Large-Scale Production and Purification of Human Lymphoblastoid Interferon	263
Borecký, L., Hajnická, V., Kontsek, P., Lackovič, V., Russ, G., Pěkníková, J., Čapková, J., Rajčáni, J., Fuchsberger, N.: Growth-Inhibitory Effect of Mouse Interferon in Transformed Cells	271
Burke, D. C.: Some Recent Advances in the Molecular Biology of the Interferon System	283
De Clercq, E.: Nucleoside Analogues as Antiviral Agents	289
De Clercq, E., Verhelst, G., Descamps, J., Bergstrom, D. E.: Differential inhibition of Herpes Simplex Viruses, Type 1 (HSV-1) and Type 2 (HSV-2), by (E)-5-(2-X-vinyl)-2'-deoxyuridines	307
Galasso, G. J.: Antiviral Agents: the Road from Scepticism to Efficacy	313
Zerial, A., Werner, G. H.: Effect of Immunostimulating Agents on Viral Infections	325

Fasciculus 4

Delgado, J. M., Herrera, L. S.: Protoplast Fusion in the Yeast <i>Candida utilis</i>	339
Nguyen Ngoc Thuy, Galgóczy, J., Novák, E. K.: Morphogenetic Effect of L-Cysteine on Dermatophytes	347
Anderlik, P., Wessely, M., Szeri, I., Bános, Zs., Rápolthy, I., Budai, J.: Formalin Stress Reaction of Germfree and Conventional Mice Treated with <i>Bordetella pertussis</i> Vaccine	359
Petrás, Gy., Bognár, Sz.: Origin and Spread of <i>Pseudomonas aeruginosa</i> , <i>Proteus</i> and <i>Klebsiella</i> during Twenty Years in an Infectious Hospital	367
Petrás, Gy., Bognár, Sz.: Extrahospital and Intrahospital Factors Predisposing to the Spread and Colonization in Patients of <i>Pseudomonas aeruginosa</i> , <i>Proteus</i> and <i>Klebsiella</i> in an Infectious Hospital	381
Kétyi, I.: Suckling Mouse Model of Urinary Tract Infections Caused by <i>Escherichia coli</i> ...	393
Sivonen, K., Szabó, I. M.: Is the Colour of the Mature Aerial Mycelium (Spore Mass) of <i>Streptomyces</i> a Diagnostic Key-Character of Decisive Taxonomic Importance?	401
Háber, K. J., Vajda, B. P., Földes, I.: Double Lysogeny of <i>Mycobacterium smegmatis</i> ...	407
Takahashi, M., Ichiman, Y., Yoshida, K.: Selection of Mouse Virulent Non-Motile Strains of <i>Escherichia coli</i> by the Soft Agar Technique	413
Fodor, T.: Note. Inhibitory Effect of Amikacin on <i>Mycobacterium tuberculosis in vitro</i> ...	419
Books Received	421

ISOLATION OF MYCOBACTERIA FROM CONTAMINATED MATERIAL ON SELECTIVE MEDIA

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(Received July 4, 1979)

A procedure more efficient than the earlier ones was developed for the isolation of mycobacteria from heavily contaminated materials. The contaminating microbes were killed by acid decontamination preceded by an incubation of 1 g sample in 5 ml nutrient broth at 33 °C for 4 h. The efficiency of isolation was examined using culture media containing varying concentrations of dimetridazole and/or clotrimazole (Canesten® Bayer). None of 40 mycobacterial strains representing all 4 Runyon groups was inhibited by 80 µg/ml of either of the inhibitors. From the sediment of acid-decontaminated samples, Löwenstein-Jensen medium containing 10 µg/ml of both dimetridazole and clotrimazole as well as Petragnani medium with and without glycerol and Šula media were inoculated. Model experiments and processing of 44 routine samples have made likely that aerobic sporeformers on the one hand and fungi and anaerobes on the other are killed, or at least depressed in growth, by pre-incubation combined with the addition of clotrimazole and dimetridazole to the culture medium. Thus, the isolation rate of mycobacteria can be improved.

Several decontaminating procedures have been developed to isolate mycobacteria from contaminated material. All are based on the resistance of mycobacteria to chemicals, e.g. sodium hydroxide and sulphuric acid [1-4]. The presence of fungi and spore-forming bacteria may, however, interfere with these procedures. Efforts have been made therefore to increase their adequacy by adding bactericidal and/or mycocidal agents to the sample or the culture medium [6-10]. Runyon's group-IV strains, especially *Mycobacterium fortuitum*, may, however, be affected by the decontamination procedures as well as by the growth-inhibitory chemicals.

Materials and methods

Pre-incubation. The multiplication phases of 16 aerobic spore-bearing strains, viz. two strains each of *Bacillus coagulans*, *Bacillus cereus*, *Bacillus megaterium*, *Bacillus subtilis*, *Bacillus pumilus*, *Bacillus sphaericus*, *Bacillus macerans* and *Bacillus polymyxa* (designations from Bergey's VIIth ed.) were determined in nutrient broth at 33 °C in the function of time.

Model experiments were designed on the basis of these experiments. One gramme of the sample was incubated in 5 ml nutrient broth at 33 °C for 4 h and then subjected to decontamination in 6% v/v H₂SO₄ for 30 min. Samples submitted to us for diagnostic purposes were processed identically.

Basal media. Šula, Löwenstein-Jensen and Petragnani media, the last one with and without glycerol, were used as basal medium.

As inhibitors dimetridazole and clotrimazole were employed, both at 40, 80 and 120 µg/ml concentration in basal media. Clotrimazole was dissolved in propylene glycol, 1 g in 15 ml, and made up to 1000 ml with 70% ethanol. In further experiments, media containing 40, 80 or 120 µg/ml of both dimetridazole and clotrimazole were used.

Strains. The 40 *Mycobacterium* strains used in the experiments are listed in Table I. The media were observed for 12 weeks after inoculation.

Model experiment. Cultures of 5 *Mycobacterium* strains, viz. *M. avium* Altman 2219 ATCC, *M. fortuitum* Weybridge 5, *M. thamnopheos* 1811 Schweiz, *M. aquae* 811, and *M. avium* III 14186-1424, were mixed to 10 pig faecal samples. One gramme of faecal sample was homogenized with 0.1 ml of culture grown on glycerol-containing Petraghani medium adjusted to 3 mg/ml density. The mixtures were incubated in 5 ml nutrient broth at 33 °C for 4 h. After acid decontamination, the samples were centrifuged at 3000 rpm for 20 min and 6 Löwenstein-Jensen media were inoculated with each. The same samples were spread on Löwenstein-Jensen media containing 20 µg/ml of both dimetridazole and clotrimazole.

For the sake of comparison, and for the evaluation of the pre-incubation procedure, faecal samples to which the given amount of bacterium had been added, were prepared for cultivation without pre-incubation, i.e. with simple decontamination. These samples were inoculated into Löwenstein-Jensen media containing 20 µg/ml of both inhibitors. The media were incubated and observed at 37 °C for 12 weeks.

Examination of routine diagnostic samples. Forty-four heavily contaminated samples were pre-incubated. Basal media (Löwenstein-Jensen, Petraghani with and without glycerol, and Šula, six of each) containing 10 µg of dimetridazole and 10 µg clotrimazole per ml were inoculated. Of the 6 parallel cultures, 2 were incubated at 28 °C, 2 at 33 °C and 2 at 37 °C. The observation period was 12 weeks.

Table I

Mycobacterium strains used in the experiments

<i>M. avium</i> I. 2: 16909-3388	<i>M. avium</i> Marway
J-2085	Dusnok 275/52
II. 2: 14141-1395	Anlich 519172
17752-372	Like 345
III. 1: 14186-1424	
IV. 2: 13528 Cheltenham	<i>M. kansasii</i>
P-55	<i>M. marinum</i>
V. 1: 12928-710	<i>M. smegmatis</i>
VI. 1: 4937	<i>M. phlei</i>
VIII. 2: 13628-1125 Davis	<i>M. thamnopheos</i> D 1811 Schweiz
14568-1686 Davis	<i>M. vaccae</i> Lábod
Altman 2219	<i>M. minetti</i>
Pe. Melnik Altman	<i>M. aquae</i> 77
P-39 Boone	81
J-2970 Boone	801
	600
Yandle	<i>M. fortuitum</i> England 5
McDarden-McAnamy	W. 4
Darden 9666/64	W. 108
Arnold Newberry	W. 487
Arnold 5808-59	77

Results

1. The growth curves for the spore-bearing strains have shown that these reached the logarithmic phase after 4 h incubation. In this phase, very few, if any, spores could be detected in the cultures, indicating that spore-formers are sensitive to decontamination in this phase.

The growth of only 5 of the 40 *Mycobacterium* strains was delayed by 120 µg/ml dimetridazole viz. of *M. avium* Altman 2219, *M. avium* Arnold 580859, *M. avium* McDarden-McAnamy, *M. avium* Darden 9666/64, and *M. avium* Arnold Newberry. The same five strains and *M. aquae* 801 were inhibited in growth by the same concentration of clotrimazole.

In the presence of both preparations at 120 µg/ml concentration, *M. fortuitum* was inhibited in addition to the above six strains.

2. Results of the model experiments are shown in Table II. The media containing 20 µg/ml of both inhibitors displayed no bacterial growth during the 12 weeks of observation following the inoculation with faecal material which had been homogenized with mycobacteria and then decontaminated. The mycobacteria added to the faecal samples could be re-isolated and identified in every case. The basal media inoculated and incubated identically remained free of microbial growth, except for 5, on each of which one fungal colony appeared after the 8th week of incubation.

Table II

Microbial contamination in media inoculated with Mycobacterium

<i>Mycobacterium</i> inoculated	D + C medium*		Basal medium		D + C medium		Basal medium	
	after pre-incubation				without pre-incubation			
	non-contami- nated	contami- nated	non-contami- nated	contami- nated	non-contami- nated	contami- nated	non-contami- nated	contami- nated
<i>M. avium</i> Altman 2219	6 6	— —	6 6	 —	5 4	— 2	1 2	5 4
<i>M. fortuitum</i> W. 4	6 6	— —	5 5	1 1	5 4	1 2	1 2	5 4
<i>M. thamnopheos</i> 1811 Schweiz	6 6	— —	5 6	1 —	6 5	— 1	2 —	4 6
<i>M. aquae</i> 801	6 6	— —	5 5	1 1	5 5	1 1	1 2	5 4
<i>M. avium</i> 14186-1424	6 6	— —	6 5	— 1	6 6	— —	— —	6 6
Totals	60	—	54	6	51	8	11	49

* D + C = basal medium containing 20 µg dimetridazole and 20 µg clotrimazole per ml

Without pre-incubation, only 11 of the media inoculated with acid-decontaminated material remained free of contamination. The others were contaminated by fungi and/or spore-bearing bacteria. Of the 5 mycobacteria, one (*M. avium* 14186-1424), owing to heavy contamination, could not be re-isolated.

Table III shows the results of the inoculations of 44 diagnostic samples on media containing 10 µg/ml of both dimetridazole and clotrimazole and the

Table III

Result of 44 diagnostic samples cultured on dimetridazole + clotrimazole and on basal medium

	Medium containing 10 µg D and 10 µg C/ml*		Basal medium	
	contaminated	non- contaminated	contaminated	non- contaminated
No. of samples yielding mycobacteria	—	552	7	545
No. of samples yielding no mycobacteria	—	504	16	488

* D = dimetridazole, C = clotrimazole

results of cultivation of pretreated samples on basal media. The isolated mycobacteria belonged to Runyon's groups I-IV.

Of the inoculated 1056 media, 23 showed contamination after the 8th week of incubation. The contaminants were fungi. No such contamination was observed on the media containing growth inhibitor.

Discussion

In the present study, decontamination combined with pre-incubation proved to be more efficient than simple acid-decontamination. Nevertheless, the results are inferior to those reported by SZABÓ *et al.* [11], who employed chlorhexidine gluconate for decontamination of mainly human sputum samples, which are known to be less abundant in microbial contamination than the samples (milk filtrates, water filtrates, stagnant water, sludge, faeces, fish-skin, etc.) usually submitted to veterinary laboratories.

Supplementing the medium with clotrimazole has proved to be advantageous. Reduction of the concentration of dimetridazole and clotrimazole to 10 µg/ml as recommended by FÜZI and CSUKÁS [2] and PLEMPER and BARTMANN [12] proved favourable; the isolation rate of mycobacteria was not influenced by these concentrations.

To the contaminations observed in the model experiments, technical errors occurring during inoculation and contaminations during incubations may have contributed.

It may be concluded that the isolation rate of mycobacteria can be increased by adding chemicals inhibiting microbial growth, even without pre-incubation, viz. to 85% compared to the 18% obtained by cultivation on the basal medium, which itself contains growth inhibitor, namely 0.2% malachite green.

According to our experience, aerobic spore-forming bacteria are partially killed by the pre-incubation procedure, but depression of fungi requires inhibitors in the culture medium. Such media are suitable for routine investigations because the viability of mycobacteria is not influenced by the concentrations employed.

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CULTIVATION OF PATHOGENIC *TREPONEMA* *PALLIDUM* IN VITRO

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Treponema pallidum was discovered relatively late and was not cultured *in vitro*. Both the delineation of *T. pallidum* biology and the eradication of syphilis suggest the necessity of cultivation *in vitro*. An attempt has been made with an improved medium to cultivate pathogenic *T. pallidum* Budapest strain *in vitro*. Only in the first passage, evidence of *in vitro* multiplication of *T. pallidum* has been established by (i) macroscopic observation, (ii) darkfield examination, (iii) electron microscopic examination, (iv) optical densities, (v) tritium labelled thymidine incorporation, and (vi) the pathogenicity of the cultured organisms was evidenced by rabbit challenge. Explanation of the oxygen utilization of *T. pallidum* suspension is discussed. Unidentified formations were observed on electron micrographs from the 96 h cultures. They may belong to the multiplication forms of treponemes. Further experiments are needed for their identification and for expansion of the multiplication of *T. pallidum* beyond the first passage.

Treponema pallidum was discovered relatively late to be the causative agent of such a serious disease as syphilis. It may be the last of the pathogenic bacteria to be cultured *in vitro*. In spite of many reports of successful cultivation, it has not been cultured *in vitro* and still remains a mystery [1, 2]. The biology of *T. pallidum* has not been delineated and its status as a bacterium or protozoan has remained in question [3]. Scientists have not agreed on the question of oxygen utilization [1, 2, 4], or on the life cycles of *T. pallidum* [1, 2]. JAHNEL [5] and MASON [6] failed to culture virulent organisms using methods previously recommended by other investigators [2]. Following their attempts there were very few reports on the *in vitro* cultivation of *T. pallidum*. Two patents were registered on techniques for the *in vitro* cultivation of *T. pallidum* in the United States by MORRISON in 1941 [7] and by ICHELSON and DENNY in 1950 [8]; however, neither was verified. More recently, FITZGERALD *et al.* [9] described their attempts to cultivate *T. pallidum in vitro* using tissue culture techniques.

There has been much interest in the successful *in vitro* cultivation of the pathogenic treponemes because it could lead to an understanding of the biology of *T. pallidum*, and possibly lead to a vaccine for immunization purposes.

It was demonstrated previously that pathogenic treponemes could divide and multiply several times *in vitro* [10]. This paper presents the results of various attempts at maintaining and extending the *in vitro* multiplication of *T. pallidum* Budapest strain.

Materials and methods

The *T. pallidum* Budapest strain used in our attempts at *in vitro* cultivation was isolated by KIRÁLY (personal communication). It was maintained in rabbits, and was used in previous *in vitro* experiments [10]. Suspensions of this strain in a defined medium were also stored frozen (-20°C) and periodically used to inoculate rabbits to verify infectivity [11].

Our basic reductive media for experiments *in vitro* were prepared in the following order. Viocin, 200 mg in 48 ml dist. water; KCl, 7600 mg in 100 ml dist. water; KH_2PO_4 , 280 mg in 50 ml dist. water; Na_2HPO_4 , 1480 mg in 100 ml dist. water; NaHCO_3 , 700 mg in 50 ml dist. water; dextrose, 1000 mg in 90 ml dist. water; MgCl_2 , 100 mg in 20 ml dist. water; Na-pyruvate, 150 mg in 20 ml dist. water; cysteine, 200 mg in 20 ml dist. water; thioglycolic acid, 0.70 ml in 50 ml 0.5% NaOH; glutathione, 400 mg in 50 ml dist. water; distilled water, 100 ml; fetal calf serum, 200 ml. The pH of the media was adjusted to 7.4 with HCl or NaOH and 0.85% NaCl solution was added to obtain a total volume of 1000 ml. All chemicals were dissolved in previously boiled and cooled (reduced) distilled water, passed through a sterile glass fritted filter by suction in the order listed and stored at -20°C in 85% N_2 –10% H_2 –5% CO_2 atmosphere.

The standard method for the routine preparation and incubation of the *T. pallidum* suspension was as follows. The treponemes were harvested from infected rabbit testes 7–9 days after inoculation with 1 ml of a suspension containing a minimum of 10^7 organisms/ml. The minced testes were placed in the basic reductive media; the flask was evacuated and equilibrated 3 times with the N_2 – H_2 – CO_2 gas and shaken on a Burrel Wrist Action shaker for 30 min. Tissue debris was removed by centrifugation for 30 min at 70 g in a refrigerated PR-6 International Centrifuge. The supernatant was diluted with media to contain 10^7 organisms/ml when examined by darkfield microscopy. The suspension was placed into sterile plastic tubes in one to four ml volumes. The tubes containing the *T. pallidum* suspension and two control tubes containing 0.02% methylene blue to detect the presence of O_2 were placed into a desiccator which was then evacuated and equilibrated three times with the gas mixture. The final pressure in the desiccator was $\text{N}_2 = 340$ mm Hg, $\text{H}_2 = 40$ mm Hg, $\text{CO}_2 = 20$ mm Hg. The routine incubation periods at 35°C were usually terminated by 168 h.

To provide evidence of multiplication, a suspension of treponemes prepared in the basic media was divided. One half was heated at 60°C for 30 min and stored at 5°C for 96 h. The other half was incubated at 35°C for 96 h. Both parts were centrifuged at 23 500 g for 30 min. The *T. pallidum* were resuspended in equal volumes of 0.85% saline, ultrasonicated, and the optical densities of each sonicate were measured at 280 nm with a Beckman Quartz Spectrophotometer.

Two other experiments were performed to determine the incorporation of thymidine by the treponemes. In the first experiment, after a preliminary incubation of 24 h, the *T. pallidum* suspension was divided. The first half was heated at 60°C for 30 min, combined with

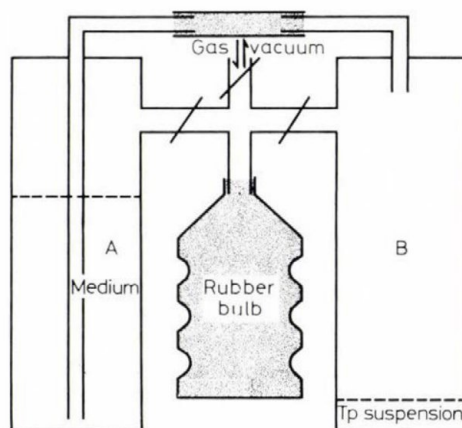


Fig. 1. Apparatus for the anaerobic cultivation of *T. pallidum* with a nonchanging atmosphere

10 μCi of tritium labelled thymidine/ml and stored at 5 °C for 72 h. The second half was combined with 10 μCi of tritium labelled thymidine and was incubated at 35 °C for 72 h. After 72 h both parts were centrifuged at 23 500 g and the organisms were washed three times with saline. The sedimented organisms were mixed with 0.4 ml NCS Solubilizar (Amersham Searle Co.) and the tritium content was determined using a Beckman Model 350 Liquid Scintillator. In the second experiment 25 μCi of tritium labelled thymidine was added to the treponemal suspension containing 10^7 organisms/ml at the time the suspension was prepared (zero hour). The suspension was divided and one half was heated at 60 °C for 30 min and stored at 5 °C for 144 h. The second part was incubated at 35 °C for 144 h. The tritium content of both parts was determined as described previously.

Large volume cultivation. Figures 1 and 2 represent equipment used in our attempts at large volume cultivation *in vitro*, i.e. 500 ml instead of one to four ml. In the first experiment (Fig. 1), the prepared *T. pallidum* suspension was placed into one flask (B) and the basic reductive medium was placed into the other flask (A). The flasks were connected by glass and Tygon tubing, rubber bulb, and stopcocks. The entire assembly was evacuated and equilibrated with the gas mixture prior to incubation at 35 °C. Each day fresh medium was forced from A to B by pressure exerted on the rubber bulb. In the second experiment (Fig. 2), the treponeme suspension was placed into chamber A and the entire unit including reservoir B and a storage flask containing new medium were evacuated and equilibrated. Every 24 h 60–70% of the spent medium was removed by pressure through a sterile glass fritted filter into the reservoir and additional fresh medium was added from the storage flask.

Figure 3 is a representation of an attempt at a combination *in vivo-in vitro* cultivation. The basic medium was passed by gravity flow through a chamber [12] implanted in a rabbit and then to a flask containing the *T. pallidum* suspension. Both flasks had been evacuated and equilibrated previously with the gas mixture. Incubation was done at room temperature (19–23 °C) for a period of 96 h.

Effect of oxygen and hydrogen. Two experiments were initiated to investigate the effect of oxygen on the survival and multiplication of *T. pallidum*. In one *in vitro* experiment, the suspension of *T. pallidum* was divided into tubes and placed into twin desiccators. Using a glass tube stopcock system (Fig. 4) both desiccators were evacuated and equilibrated 3 times with the $\text{N}_2\text{-H}_2\text{-CO}_2$ gas mixture then one desiccator was subsequently inoculated with 0.5 ml of air each day. Incubation was done at 35 °C for 96 h. In the experiment *in vivo* subcutaneous culture chambers [12] were implanted in mice. The suspension of *T. pallidum* was divided into equal doses, the chambers of one set of mice were inoculated with *T. pallidum* followed

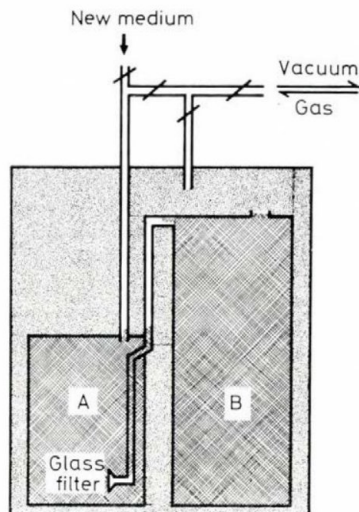


Fig. 2. Apparatus for the anaerobic cultivation of *T. pallidum* with the potential for exchanging medium under anaerobic atmosphere. Each day removed 60–70% of the spent culture medium from A to B and an equal volume of new culture medium was replaced into A

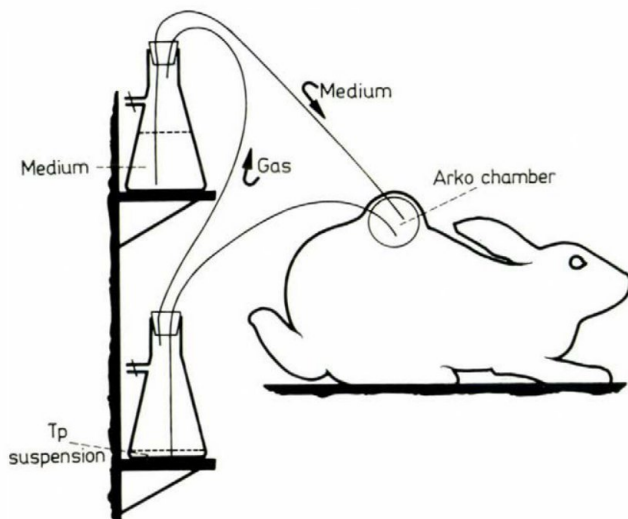


Fig. 3. Combination of *in vitro* and *in vivo* methods for cultivation of *T. pallidum*

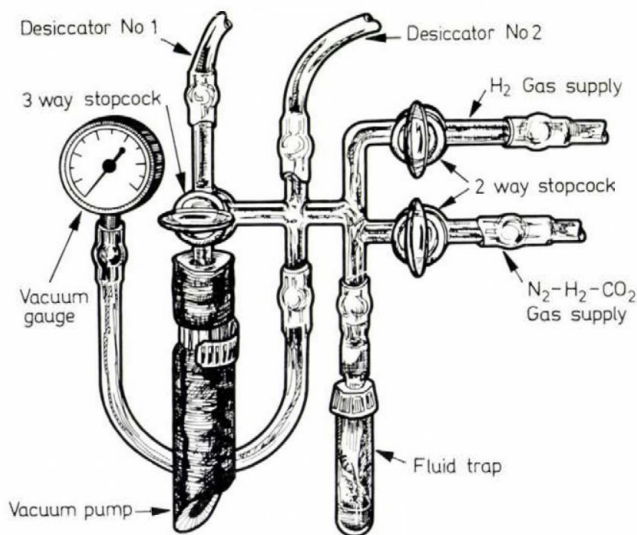


Fig. 4. Glass tubing stopcock system

by the injection of 1 ml O₂. The second set of chambers was inoculated with *T. pallidum* only. The presence and survival of the treponemes were determined by darkfield examination of fluid withdrawn periodically from the chambers.

In another twin desiccator experiment to evaluate the effect of atmospheric conditions, one desiccator was evacuated and equilibrated three times with ultrapure (99.99%) H₂ gas. The experiment was assessed by darkfield examination for the presence and survival of treponemes exposed to H₂ only and to the N₂-H₂-CO₂ gas mixture.

To determine the influence of human trace elements [13, 14] on the survival of *T. pallidum*, the following chemicals were investigated: CdSO₄, CoSO₄, CuSO₄, NiSO₄, ZnCl₂, BaSO₄, LiNO₃,

MnSO₄, SnCl₂, CaCl₂, NaBr, K₂Al₂(SO₄)₄, KI, and FeCl₃. All chemicals were dissolved in 0.85% saline to an initial concentration of m/100. Further subdilutions were prepared as needed. All chemicals were added at a ratio of 0.1 ml/ml *T. pallidum* suspension. Results of the additions were observed by darkfield examination daily. Survival of the treponemes was recorded in terms of per cent motility.

The electron microscopic examinations included whole mount preparations and ultrathin sections. For whole mount preparation the culture tubes were vigorously shaken with a Vortex-Genie to dissociate the treponemal floccules as much as possible. A drop of this suspension was placed on Formvar coated copper grids, blotted dry and stained negatively with 2% uranyl acetate, pH 3.8. For thin sections the floccules were fixed in 2% glutaraldehyde in 0.1 M sodium cacodylate buffer pH 7.4, for two hours at room temperature. The suspension was centrifuged at 35 000 g in a Sorvall Superspeed RC2B Automatic Refrigerated Centrifuge and the pellet washed in a 0.1 M cacodylate sucrose buffer rinse at 4 °C overnight. The pellet was postfixed in buffered 1% osmium tetroxide, dehydrated in graded ethanols, infiltrated with propylene oxide and embedded in Maraglas. The specimen blocks were sectioned with a Servall Porter-Blum Mt-1 ultramicrotome. Then sections were picked up on naked copper grids and double stained with saturated uranyl acetate in methanol and aqueous lead citrate [15]. The whole mounts and thin sections were examined with a Philips Model 200 transmission electron microscope operating at accelerating voltages of 40 and 60 kV.

Comparison of the Budapest and Nichols strains was made using fluorescent antibody techniques [16, 17] and a modified micro haemagglutination technique [18].

Periodically to verify virulence, a sample of treponemes incubated for 168 h was inoculated intratesticularly into rabbits (1 ml/testis).

Results

The results of our experiments were assessed by one or a combination of: (i) macroscopic observations, (ii) darkfield examinations, (iii) electron microscopic examinations, (iv) optical densities, (v) tritium labelled thymidine incorporations, and (vi) the pathogenicity of the cultured organisms as evidenced by intratesticular infection with 1 ml of culture material/rabbit testis. These are shown in graphs, photographs, darkfield micrographs, and electron micrographs.

Evidence of multiplication occurring during incubation of the small tubes of the treponemal suspension was obtained by darkfield examination. Figure 5 shows that a *T. pallidum* is dividing by transverse fission after 24 h incubation as shown previously [10]. Both treponemes showed rotation in opposite directions and the midpoint (arrow) shows two tapering ends rather than the dense bud commonly observed when two treponemes are attached to each other. Figure 6 shows the start of floccule formation and another dividing form. Increase in the size of the floccule at 48 h is evident in Fig. 7. Combination of small floccules into larger units by 72 h is shown in Fig. 8. The difficulty of counting the number of treponemes accurately is evident from Fig. 9 which shows uncountable numbers of *T. pallidum* to be caught up within the floccule. An accurate and reproducible count of the free treponemes was not possible due to the presence and various size of the floccule when the slides were prepared for counting. It was, however, the impression of the observers that as the floccules increased in size, the number of three *T. pallidum* seemed to remain constant. By 96 h of incubation the floccules were easily

visible macroscopically, being approximately 9% of the total volume of the tube as is seen in Fig. 10 (arrows). Figure 11 is a darkfield micrograph of a small portion of the floccules seen at the bottom of the tube in Fig. 10.

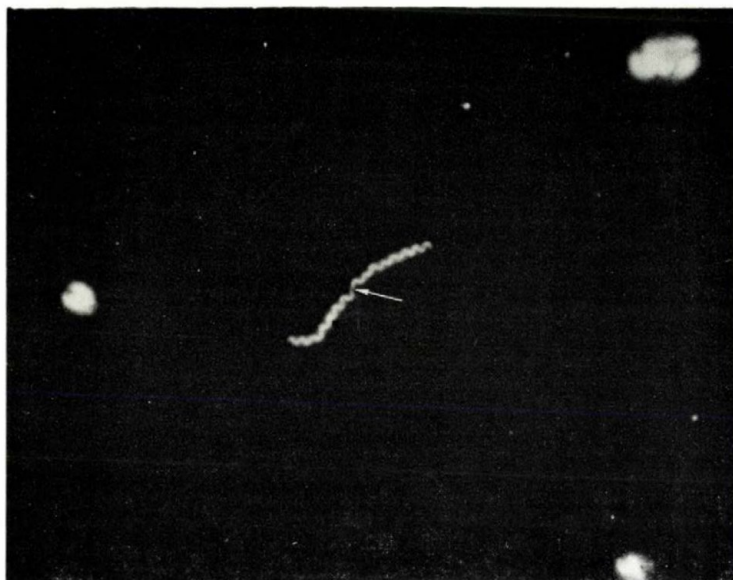


Fig. 5. Dividing form of *T. pallidum* in 24 h culture. $\times 1000$

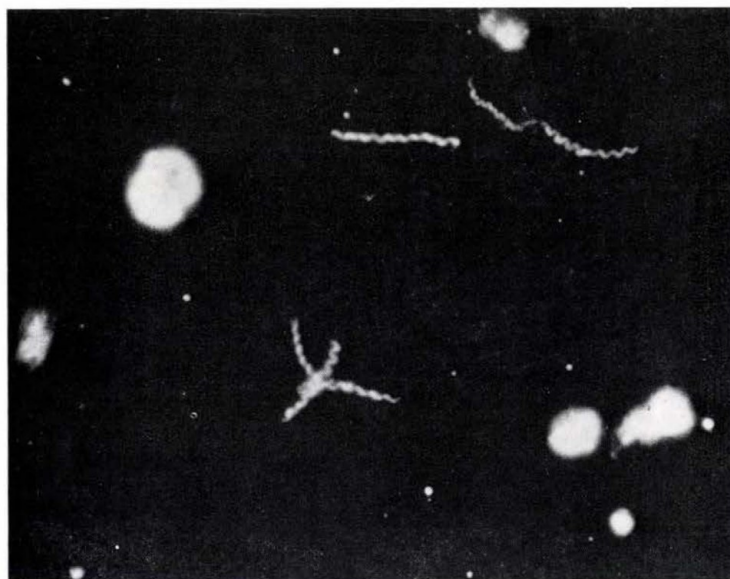


Fig. 6. Dividing form of *T. pallidum* and beginning of floccule formation. $\times 1000$

Further evidence of multiplication was obtained by adsorption measurements (Fig. 12) and thymidine incorporation (Fig. 13). In the absorption experiment the 37 °C culture showed an increase in protein concentration whereas



Fig. 7. Small floccules from 48 h culture. $\times 1000$

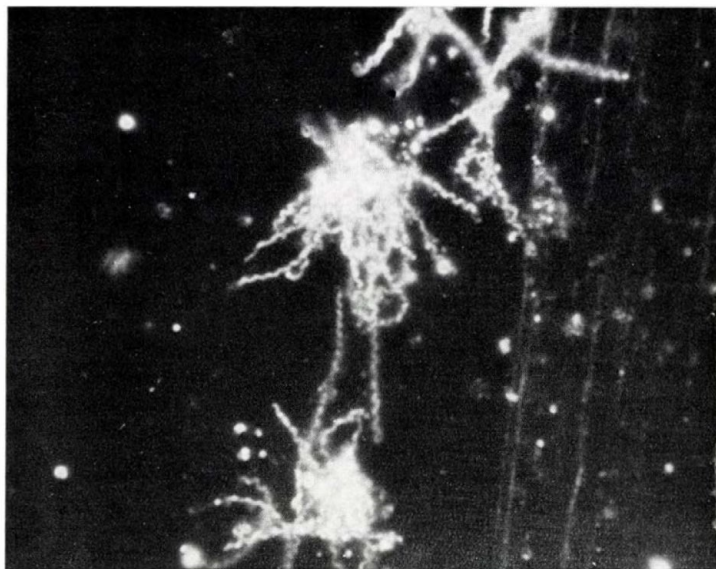


Fig. 8. Aggregation of small floccules from 72 h culture. $\times 1000$

the heat killed (60 °C) control remained at the initial optical density level. In both thymidine experiments a statistically significant incorporation was obtained in the 37 °C culture treponemes as compared to the heat killed (60 °C) controls.

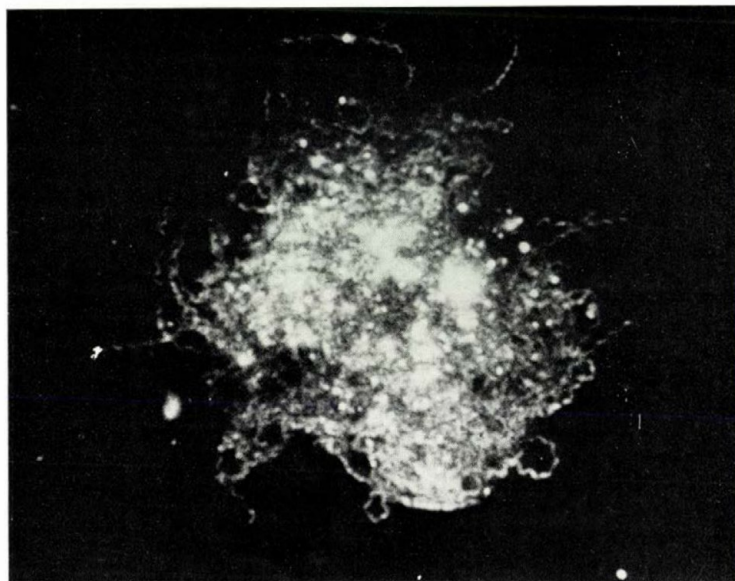


Fig. 9. Uncountable numbers of *T. pallidum* composing floccule. $\times 1000$

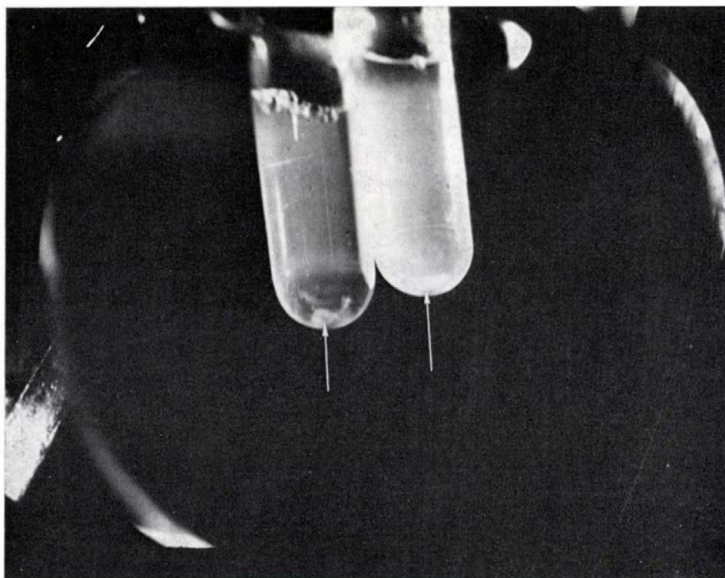


Fig. 10. Photograph of 96 h floccules (arrows) at the bottom of the tubes

Results of using human trace elements as supplements to the basic media (Table I) showed that tin ($LD_{50} = M/100$) had a beneficial effect on survival of the treponemes as compared to the treponemes cultured in the basic media alone. Copper ($LD_{50} = M/8600$) was the most detrimental element investigated.

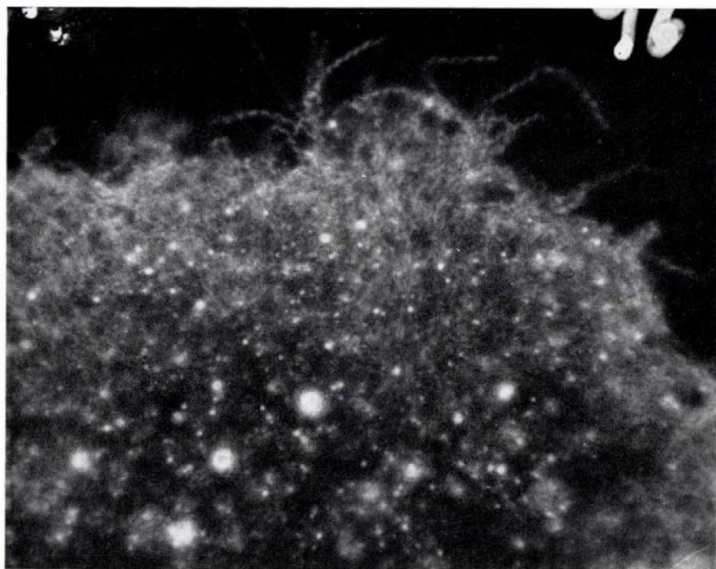


Fig. 11. Small section of floccule of 96 h culture. $\times 1000$

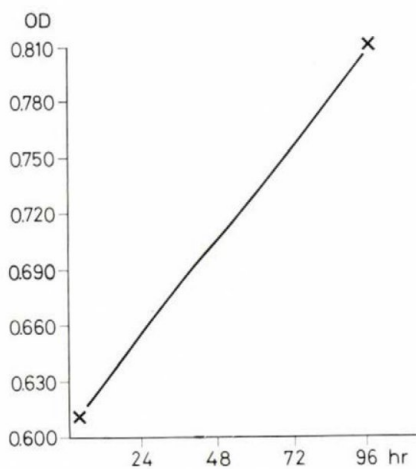


Fig. 12. Optical density (OD) of *in vitro* cultivated *T. pallidum* after sonication (280 nm)

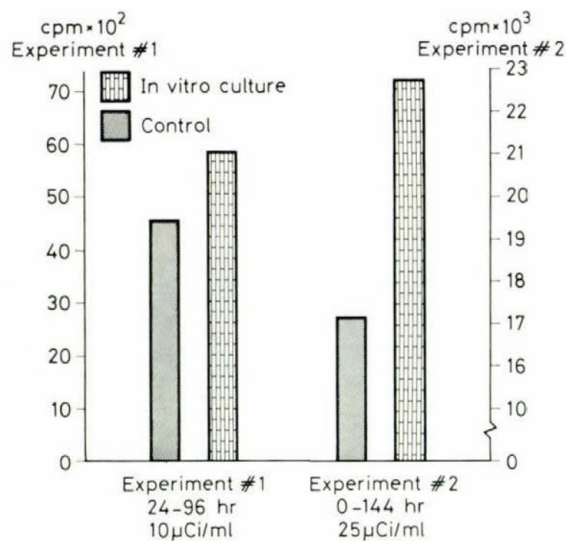


Fig. 13. Results of thymidine incorporation

Table I

Incubation time for 50% lethal dose (LD_{50}) of trace elements

Chemicals	Incubation time in hours			
	72*	96*	120**	192**
CdSO ₄		M/900		
CoSO ₄		M/300		
CuSO ₄			M/8600	
NiSO ₄			M/100	
ZnCl ₂			M/900	
BaSO ₄				M/100
LiNO ₃				M/100
MnSO ₄				M/100
SnCl ₂				M/100***
CaCl ₂		M/100		
NaBr		M/100		
K ₂ Al ₂ (SO ₄) ₄		M/100		
KI		M/100		
FeCl ₃		M/100		

* Less than 50% immobilization.

** Greater than 50% immobilization.

*** 97% motility at 168 hours in one experiment.

Electron micrographs of the floccules showed individual treponemes (Fig. 14), apparent coccid formations of treponemes (Fig. 15), and other material. Some forms looked like cross sections of bundles of treponemes surrounded by a partial membrane (Fig. 16) or within an intact membrane (Fig. 17). These formations were observed in many areas and were the most dominant feature in the floccules. Figure 18 shows numerous internal membranes (possible *T. pallidum*) surrounded by an outer membrane and Fig. 19 shows an ultrathin section of rabbit mitochondria and treponemes from a 96 h culture. Figure 20 shows an ultrathin section from a 96 h culture with two longitudinal sections of treponemes and membranous material which appears to be a cross section of a number of *T. pallidum* surrounded by a membrane.

In mouse experiments *in vivo* it was demonstrated by darkfield examination of chamber fluid that compared to the controls 90% of the treponemes disappeared or died in the oxygen environment.

Darkfield examination of tubes from the first twin desiccator experiment in which one desiccator was injected with 0.5 ml of air did not show a significant difference in number or motility of the organisms at the end of 96 h of incubation. In the second twin desiccator experiment the number and motility of the *T. pallidum* were reduced in an atmosphere of H_2 as compared to the treponemes exposed to the $N_2-H_2-CO_2$ gas mixture.

The 168 h cultured Budapest strain was comparable to the Nichol's pathogenic strain obtained from rabbit testicular material in both the direct and indirect fluorescent antibody techniques and as a source of antigen for the *T. pallidum* haemagglutination procedure.

In the two experiments using large volumes of media, multiplication and formation of floccules was not observed.

The *in vivo-in vitro* experiment was considered a partial success as small floccules were observed macroscopically in the flask containing the treponeme suspension prior to the unintentional termination of the experiment. Between 72 and 96 h of observation, the rabbit had chewed the tubing apart and bacterial contamination was observed at 96 h.

Periodically, after 168 h incubation in the plastic a sample of the treponeme suspension was inoculated intratesticularly into rabbits to verify the retention of virulence. A syphilitic orchitis was observed 8 to 12 days post-inoculation.

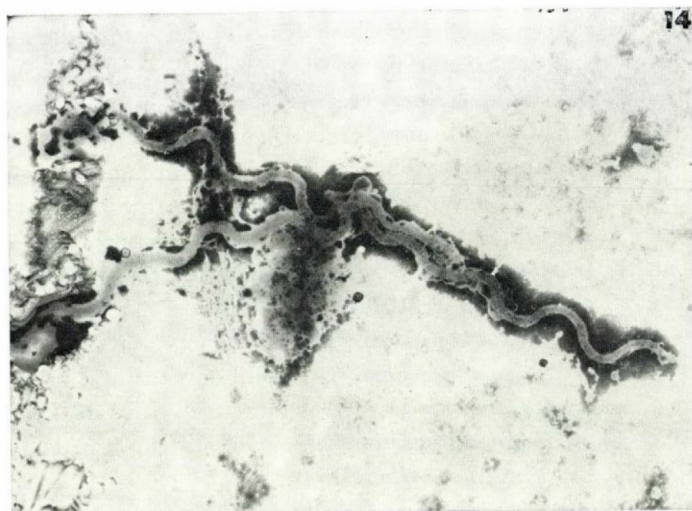


Fig. 14. Electron micrograph of negatively stained treponemes from a 96 h culture.
 $\times 18\,798$



Fig. 15. Ultrathin section of the coccid formation of treponema.
 $\times 67\,960$



Fig. 16. Portion of treponemes surrounded by partial membranes.
 $\times 76\ 860$



Fig. 17. Portion of treponemes within an intact membrane (arrows).
 $\times 153\ 798$

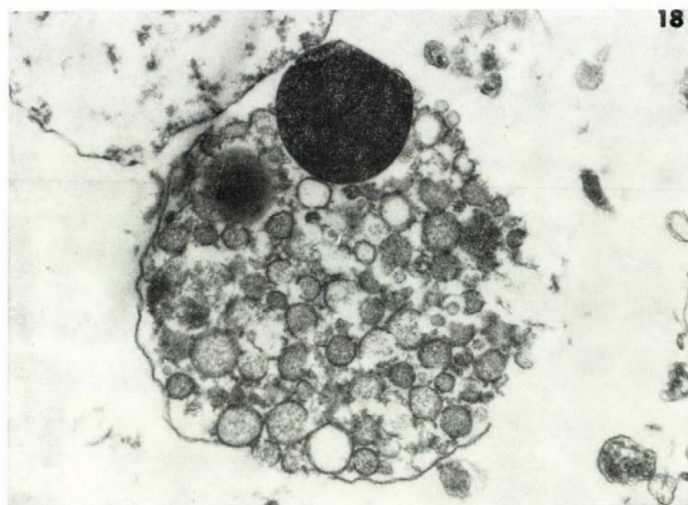


Fig. 18. Unidentified material from 96 h culture surrounded by a membrane.
×38 834. Similar to Fig. 20

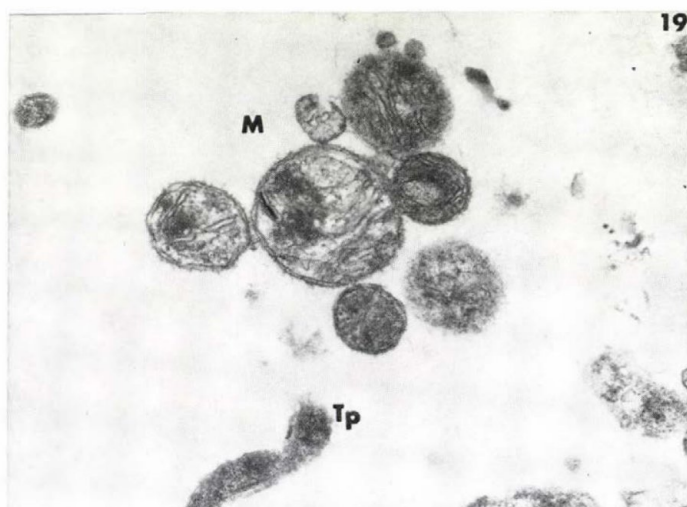


Fig. 19. Ultrathin section of rabbit mitochondria (M) and treponema (Tp) from 96 h culture. ×58 144

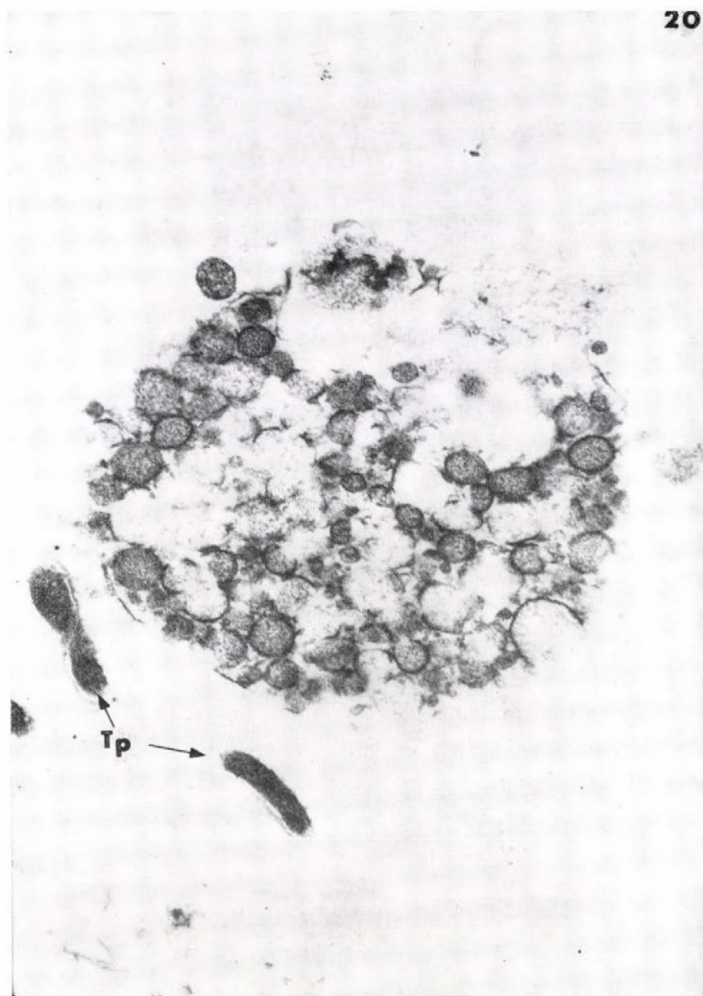


Fig. 20. Ultrathin section from 96 h culture with two longitudinal sections of treponemes (Tp) and unidentified material which appears to be a cross section of a number of treponemes surrounded by a membrane. $\times 52\ 875$

Discussion

When reporting the results of our experiments, we hope to encourage future investigations into the cultivation of pathogenic *T. pallidum* which will give consistent multiplication without loss of virulence, motility, morphology, antigenicity and pathogenicity.

In our experiments using defined conditions of atmosphere, media, temperature, and time we have been able to demonstrate that *T. pallidum* (a) remain viable for a limited time; (b) multiply to a certain extent; (c) retain their typical morphology; (d) retain their virulence for animals; and (e) retain certain antigenic properties.

Using the standard method described previously, we were able to repeat results of incubation on a weekly basis. Formation of floccules was observed macroscopically as well as by darkfield microscopy, and as floccules developed and increased in size the number of free *T. pallidum* appeared to remain constant. The 168 h culture material from the tubes was repeatedly used to inoculate rabbits intratesticularly with infections developing. Even though the medium is highly reductive, the atmosphere under which the treponemes are maintained was very important, as shown first by accident and then partially by experimental design. Occasionally, after the *T. pallidum* had been incubated for 24 h, the methylene blue control tubes demonstrated that oxidation had occurred. Darkfield examination of the suspensions of *T. pallidum* revealed complete loss of motility and they were discarded. In an attempt to show this phenomenon by design, the first twin desiccator experiment was initiated. Although there was not any detrimental effect on the experimental suspension when compared to the suspension under standard conditions, the amount (0.01 ml) of O_2 inoculated was probably too small to overcome the reductivity of the medium. The mouse experiment *in vivo* gave dramatic results when a larger per cent of O_2 was introduced into the culture chambers. Results of the human trace element study would tend to support the role of reductivity since the greatest survival was detected in the medium containing stannous chloride, which is highly reductive. The results obtained in the presence of O_2 conflict with the theory proposed by COX and BARBER [4] on the utilization of O_2 by treponemes. Only under strict anaerobic conditions did our standard cultivation succeed.

We also have electron microscopic evidence that in preparing the treponeme suspension a great number of mitochondria were liberated from the cells of the infected testes (Fig. 19). An acceptable explanation of the oxygen utilization might be due to this large number of mitochondria since it has been shown that oxygen consumption in mitochondria is about 5 times higher than in a suspension of tissue homogenate [19]. It is, however, also conceivable that the unsaturated lipid acids and the sulphydryls of *T. pallidum* can take up

oxygen. How fast this oxidation takes place is unknown but because of the large surfaces involved it would be expected to occur very rapidly. It is possible that oxidation prohibits the cultivation of *T. pallidum in vitro* as it has been shown that varying the duration of O₂ exposure can hinder the cultivation *in vitro* of other anaerobic microorganisms.

The results of the two thymidine uptake experiments and the absorption experiment are proof that multiplication did occur for a period of time. This evidence would complement the darkfield observations of the replicating forms and formation of the floccules without apparent decrease in free *T. pallidum*.

Why multiplication ceased and our attempts at large volume cultivation *in vitro* failed is not known. It seems that both phenomena occurred because of changes associated with the medium. It may have become toxic first, due to the accumulation of metabolic end-products; second, due to a decrease in one or more of the essential nutrients; third, due to the reductive power having been altered in some manner, or due to a combination of these. It appears from the results of the O₂ effects and those obtained in the human trace element experiment that the greater or higher the reductivity of the media, the greater the chances for longer survival and, possibly, the greater the chances of cultivation.

Future experiments are planned for maintaining and increasing reductivity of the medium; investigating other chemicals which have been suggested as being beneficial to the survival of *T. pallidum*, and for neutralizing the toxicity of the metabolic end-products.

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A SIMPLE PROCEDURE FOR ISOLATION AND PURIFICATION OF A-TYPE STAPHYLOCOCCUS ENTEROTOXIN*

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A new procedure is presented for the isolation and purification of A-type staphylococcus enterotoxin. Homogeneous enterotoxin preparation was obtained by purification in 2 phases. In radial double agar-gel immunodiffusion the smallest precipitating dose of the isolated and purified enterotoxin was found to be 1.4–0.7 μg protein and 0.4–0.1 μg nitrogen. In cat experiments the dose giving a positive reaction was 2 μg protein or 0.5 μg nitrogen calculated for kg body weight.

On the basis of their serological characteristics, enterotoxins produced by enteropathogenic staphylococci belong to groups designated with capital letters from A to F [1]. Toxins designated with different letters differ from each other not only as antigens but also in several physico-chemical features, though their biological effects are practically identical [2, 3].

Several procedures have been elaborated for the isolation and purification of serologically different enterotoxins. These procedures have several stages which differ from each other in their complexity. Two research groups prepared from A-type enterotoxin homogeneous preparations. SCHANTZ *et al.* [4] obtained 20% recovery with a 4-stage procedure. Similarly, the 4-stage method of EZEPCHUK *et al.* [5] resulted in 30–40% recovery. A 2-stage procedure is described here which allowed 50% recovery of the active material in homogeneous preparation.

Materials and methods

Organism. FRI 722(H) strain was used to produce the A-type enterotoxin.

Medium. Casman's fluid culture medium in the modification of BUGROVA [6] was used for cultivation. Instead of the HCl, an enzyme digested casein hydrolysate (produced in the USSR) was used in the culture medium. The end concentration of the non-protein nitrogen was 150–160 mg/100 ml in the medium. The 18 h preculture on slant MPA broth-peptone agar of the standard strain was given to 1 litre of modified Casman medium pH 7.1–7.2, in 5 litre flasks with flat bottom. Culturing with aeration was performed at 37 °C for 22–24 h in a shaker at 120–150 rpm, and the bacterial cells were separated by centrifugation at 3500 rpm. Precipitation with ammonium sulphate of analytical purity was applied for isolation of the toxin. Further purification was carried out on DEAE cellulose (Sigma) balanced with 0.005 M Tris-HCl buffer (pH 7.2).

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Protein determination was performed according to LOWRY *et al.* [7]. Nitrogen was estimated by a micro-technique using Nessler reagent, at 420 nm wavelength [8]. Polyacrylamide-gel disk electrophoresis in SDS system was carried out according to WEBER and OSBORN [9].

Radial double agar-gel immunodiffusion according to OUCHTERLONY [10] was used to determine the presence of the toxin and the titre value, and on its basis the precipitate-forming lowest amount of nitrogen, i.e. the toxin unit (U). The biological effect of enterotoxin was studied in cats of 3.0–3.5 kg weight. The purified preparation was diluted with physiological saline to different protein concentrations and 0.5–1.0 ml of the dilutions was introduced in the femoral vein of the animals. Vomiting and diarrhoea were considered a positive reaction, faintness, shaking chill and diarrhoea as uncertain, and the lack of these symptoms as negative [11].

Results

The aim of the studies was to find a procedure which provides the best recovery rate of the toxin from the culture filtrate in concentrated and appropriately purified form. Three earlier methods: sorption-elution on Amberlite CG-50 ion exchange resin [4], lyophilization [12], and ammonium sulphate precipitation [13] have been compared. As shown in Table I, the best results of A-type enterotoxin isolation from the culture filtrate were obtained by ammonium sulphate precipitation. The homogeneity of the toxin showed a 120-fold increase and its recovery was 75–80% with precipitation. Amberlite CG-50 sorption ensured a similar homogeneity but only a 37–40% recovery rate. Lyophilization gave unsatisfactory purity. In further studies, recovery of the toxin was examined according to the ammonium sulphate concentration. As shown in Table II, no toxin activity was observed at 40% concentration. As shown by agar gel immunodiffusion, the active material was recovered in 60–80%.

Table I

Concentration and primary purification methods of A-type staphylococcus enterotoxin

Concentration of the material	Volume (ml)		Total nitrogen, mg/ml	Activity			Specific activity, U/mg N	Purification ×	Recovery, %
	before treatment	after treatment		agar-gel diffusion titre	lowest precipitating dose, U/mg N	Unit in 1 ml			
Native culture	900	—	4.0	1/4	0.017	235.3	58.8	—	—
Lyophilization	100	40	6.0	1/8	0.0127	472.4	78.7	133	75–80
Ammonium sulphate (60%) precipitation	1400	35	1.0	1/128	0.00013	7692.4	7693.3	130.8	75–80
Sorption on Amberlite CG-50, elution	400	40	0.12	1/16	0.000127	944.8	7874.0	133.9	37–40

Table II*Recovery of A-type staphylococcus enterotoxin in the function of ammonium sulphate saturation*

Saturation with ammonium sulphate (%)	Index of enterotoxin concentration (×)	Activity (titre with agar gel immunodiffusion)
0 (native filtrate)	1	1/4
40	10	not observable
60	10	1/64
80	10	1/64

Sedimentation with 60% ammonium sulphate was applied in the first phase of the procedure. The dissolved and concentrated precipitate was washed with flow-through dialysis until the SO_4 ions were eliminated, as controlled with barium chloride. Following dialysis, the undissolved contaminating materials were eliminated by centrifugation. In the second phase, the dialysed material was treated with DEAE cellulose. One weight unit of crude cellulose balanced with 0.005 M Tris-HCl buffer (pH 7.2) was added to one volume of the dialysed material (e.g. 50 ml material + 50 g cellulose). The two materials were mixed thoroughly in a cup for 10–15 min, and centrifuged at 6000 rpm for 25–30 min to separate the cellulose. Agar-gel immunodiffusion revealed the localization of the enterotoxin in the colourless supernatant. Consequently, the contaminating materials had become adsorbed to the cellulose, but not the toxin. Detailed data are presented in Table III.

With the volume unchanged the quantity (titre) of the enterotoxin decreased slightly after sorption as revealed by agar-gel immunodiffusion.

Table III*Purification of A-type staphylococcus enterotoxin by ammonium sulphate precipitation and subsequent DEAE cellulose treatment*

Characteristics of the toxin	Crude culture	After ammonium sulphate precipitation	After DEAE cellulose sorption
Volume, ml	900	45	45
Total nitrogen, mg/ml	4.5	1.6	0.3
Titre, agar-gel immunodiffusion	1/4	1/64	1/32
Lowest precipitating dose, mg N/U	0.02	0.00042	0.00016
Specific activity, U/mg N	50	2380.9	6250.0
Purification, ×	—	47.6	125.0
Recovery, %	—	84.6	41.6

In contrast, the specific activity of the toxin increased significantly. As compared to the culture filtrate, the purity of the toxin was 125-fold, in some series 250-fold. Enterotoxin recovery was 40–45%. In disk electrophoresis (SDS system) the isolated and purified A-type enterotoxin occurred in one zone; this proved the homogeneity of the recovered protein (Fig. 1).

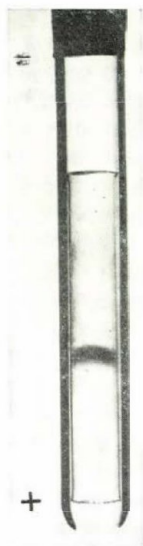


Fig. 1. Disk electrophoresis of A-type enterotoxin obtained by two-phase purification

In disk electrophoresis, using diphtheria toxin and its fragments of known molecular weight as indicator protein, the isolated and purified A-type enterotoxin had a molecular weight of 27 500 dalton. The isolated and purified enterotoxin exerted a strong biological effect in the cat (Table IV). The minimum toxic dose of the preparation has not been determined reliable; it was, however, found that intravenous administration of 2 μ g toxin in 1 ml volume caused acute vomiting after 30–40 min.

Table IV
Effect of purified A-type staphylococcus enterotoxin in cats

Preparation	Dose μ g protein/kg body weight	Dose, ml	No. of cats	Evaluation of the reaction
Series 1 (material obtained by cellulose treatment)	10	1.0	2	+
	5	1.0	2	+
Series 2 (material obtained by cellulose treatment)	10	1.0	2	+
	2	1.0	2	+

Discussion

A-type staphylococcus enterotoxin can be obtained in purified form with different methods. The different phases of the methods decrease, however, significantly the quantity of recoverable toxin. This is one of the facts that hindered a more detailed study of the structural differences of A-type enterotoxin. The two-phase method described provided means to overcome this difficulty and to obtain a significant quantity of active material. The first phase consists of ammonium sulphate precipitation. This simple procedure allows to isolate large amounts of enterotoxin. The second phase, DEAE cellulose treatment is also simple and easy to carry out, but causes a decrease in titre of the enterotoxin as some of the toxin is adsorbed to the cellulose. As the titre of the enterotoxin did not decrease parallel with the increase in the quantity of cellulose used for the elimination of the ballast material, one may assume the presence of two toxin types. One of them adsorbs to cellulose balanced with 0.005 M Tris-HCl buffer pH 6.8-7.6, while the other one remains in solution.

Some data characterize the A-type staphylococcus enterotoxin as a protein of two different molecular weights, of 28 000 and 32 000-34 000 dalton [2, 5]. A realistic assumption is that the toxin of 32 000-34 000 dalton molecular weight adsorbs to cellulose. The 28 000 molecular weight toxin failed to adsorb to the cellulose. If the separation of the two toxins different in molecular weight can be proved, then they have to be subjected to special studies. The deviating chromatographic characteristics of A-type staphylococcus enterotoxins are indisputable.

Disputing of purified toxin it is our aim to prepare monovalent immune sera, the specificity of which can be compared with that of the polyvalent immune serum produced against non-purified toxin, or with that of the specific immune serum of Serva (FRG).

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ISOLATION AND MUTATION OF CELLULOLYTIC FUNGI

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Nineteen fungi were isolated from different soil samples on the basis of clear zones formed on Rose Bengal Cellulose agar medium. In shake flasks the isolate K₁ gave 12.1 units/ml of CMCase activity. A mutant of the isolate K₁, KM₇, was selected after *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine treatment of the wild-type. This mutant differed morphologically from the parent strain on RBCA medium and gave 36.2 units/ml of CMCase activity which represented about 50% of the enzyme yield from the standard organism, *Trichoderma viride* QM 9414 (80 units/ml of CMCase activity). The isolate K₁, which was identified as a *Phoma* species, produced 48 units of β -glucosidase. The yield of β -glucosidase was increased about 8-fold in the mutant KM₇ and was about 68% higher than the level found in *T. viride* QM 9414.

The search for organisms producing active cellulases has led to the discovery of cellulolytic fungi like *Trichoderma viride*, *Sporotrichum pulverulentum* and *Fusarium solani*. These organisms are truly cellulolytic, i.e. they have all the enzyme components needed for the degradation of crystalline cellulosic materials. Some pseudocellulolytic organisms are also known, which grow only on reactive celluloses and are lacking the C₁ component of the cellulase complex. Attempt to improve the fermentation conditions and to develop mutant strains have resulted in greatly increased enzyme yields. *Trichoderma reesei* NG-14, a mutant of *T. viride* QM 6a, produced at the Department of Biochemistry and Microbiology, Cook College, Rutgers University, U.S.A., is reported to give the best enzyme yields [1].

The main objective of the present study was to isolate and mutate a cellulolytic fungus which could produce active cellulase enzyme for the saccharification of waste cellulosic materials.

Materials and methods

Isolation. An attempt was made to standardize a procedure for the quick screening of isolation of the cellulolytic fungi from soil samples. Rose bengal cellulose agar medium (RBCA) was prepared after BOSE [2], and OTTOW [3]. This medium has the characteristic of preventing the spread of fungal colonies and therefore their size is limited. The medium is composed of sodium nitrate, 2.0 g; potassium dihydrogen phosphate, 1.0 g; magnesium sulphate (heptahydrate), 0.5 g; potassium chloride, 0.5 g; ferrous sulphate (heptahydrate), 10.0 mg; agar (Difco), 1.5%, and acid precipitated cellulose, 1.0% per litre. Rose bengal (50 μ g/ml) added to the above medium helps in restricting the size of fungal colonies. The acid precipitated cellulose was prepared as follows. Small strips of filter paper (Whatman No. 1) were immersed in concentrated hydrochloric acid at a ratio of 3 g/100 ml at 25–27 °C for 3 h with occasional shaking. The mixture was then poured under stirring into an excess of distilled water and kept

there for 2-3 h. The supernatant was poured off, the residue washed repeatedly with distilled water in a Buchner funnel until free from acid, washed with 95% ethanol air dried and ground in a mortar.

The cellulose powder was soaked in a few ml of screening medium (without agar and Rose bengal) and made into a fine paste in a mortar. The remaining medium was then added to the required volume and, after addition of Rose bengal and agar, sterilized by autoclaving.

One ml of the filtered soil suspension was spread onto plates of solid medium and incubated at 28 °C. Colonies showing clear zones around them after 3-4 days of incubation were transferred to potato dextrose slant agar (PDA). These organisms were again tested for their capacity to produce clear zones on RBCA plates and the pure colonies thus obtained were picked up and maintained of PDA slants.

Mutation with N-methyl-N'-nitro-N-nitrosoguanidine (NTG). The isolate K₁ which gave highest CMCase activity in shake cultures was treated with NTG to produce mutants with higher yields of CMCase. A fresh growth of isolate K₁ on PDA was used and the spores were suspended in 10 ml of sterilized distilled water containing 0.1% Tween-80. One ml of the spore suspension was plated onto RBCA medium to obtain the initial count. To the remaining spore suspension, NTG solution prepared in acetone was added to give a final concentration of 200 µg/ml. The contents were mixed well, incubated at room temperature and one ml samples were removed at different intervals. In order to stop the action of NTG, 10 mg of cysteine was dissolved in the first tube which subsequently was serially diluted and plated for counting of the survivors.

Enzyme preparation and assays. The isolates giving clear zones on RBCA medium were grown in liquid culture on REESE and MANDELS [4] medium to determine CMCase by the method described previously [5]. The culture filtrate of the mutant KM₇ was assayed additionally for β-glucosidase and Avicelase as follows.

β-Glucosidase activity [6]. β-Glucosidase activity was determined by a modification of Norkans method using p-nitrophenyl-β-D glucoside as substrate. The assay mixture contained 1.0 ml of mM p-nitrophenyl-β-D glucoside in 0.05 M sodium acetate buffer pH 5.0 and 100 µl enzyme solution. After incubation at 40 °C for 10 min, 2.0 ml 1 M Na₂CO₃ was added to the mixture to stop the reaction. The mixture was diluted with 10.0 ml of distilled water and the p-nitrophenol liberated was determined from the absorbance at 400 nm. The standard curve was prepared from the stock solution of p-nitrophenol (80 µg/ml). The unit of enzyme activity was defined as the amount of enzyme which liberated 5 µg p-nitrophenol under the assay conditions.

Avicelase activity [6, 7]. The reaction mixture consisted of 2.0 ml of a 1% suspension of Avicel RC 581 in 0.05 M sodium acetate buffer (pH 5.0) and 200 µl of enzyme solution. After incubation at 30 °C for 2 h, the mixture was filtered (Whatman filter paper No. 1) and analysed for reducing sugars by the method of MILLER [8].

One unit of enzyme activity was defined as the amount of enzyme that liberated reducing sugars corresponding to 5 µg glucose under the assay conditions described above.

Results and discussion

Isolation of cellulolytic fungi from soil. When solid media are used for the isolation of fungi from soil, the main problem is the spread of colonies over the plates. In order to restrict the growth, which in turn results in accumulation of metabolic products including enzymes around the colonies, OTTOW [3] recommended the use of Rose bengal in the solid medium. MONTENECOURT and EVELEIGH [1] used Rose bengal (50 µg/ml) for the screening of cellulolytic fungi. Using Rose bengal cellulose agar (RBCA) medium, 19 organisms were isolated; they all showed clear zones around their colonies. These isolates were further purified by repeated subculturing. Their capacity to produce carboxymethyl cellulase (CMCase, Cx) was determined after growth in shake flasks on REESE and MANDELS medium [4]. Data on enzyme activity, colour of spores, and the final pH of the filtrates, are presented in Table I.

Enzyme activities varied between 0.71 units to 12.1 units per ml. In all cases an acid final pH of the filtrate was observed. The isolates K₁, K₂, K₃ (with black, grey and black spores, respectively) gave high activities ranging from 8.5 to 12.1 units/ml. There was no clear correlation between the final pH and enzyme activity. Similarly, there was no definite relationship between the size of clear zones on RBCA medium and the CMCase yield in the shake flasks. An isolate (K₄) with a sharp clear zone gave only 4.8 units/ml of activity. Isolates K₁ and K₂ were exceptional in giving good clear zones as well as high enzyme yields.

In the case of *T. viride* QM 6a, MONTENECOURT and EVELEIGH showed that the increased enzyme production capacity was reflected by the formation of zones of different size [1]. This might have been due to the presence of phosphon-D, which they included in the solid medium and which is known to facilitate the release of enzymes.

The isolate K₁ gave the highest enzyme yield and was selected for further study.

Table I
Carboxymethyl cellulase (CMCase) activity of soil isolates

Isolate	Colour of spore	CMCase activity (units/ml)	Final pH of filtrate
C ₁	Yellow	4.2	5.1
C ₂	Light blue	4.1	5.0
C ₃	Yellow	4.0	5.2
C ₄	Pink	1.2	6.1
C ₅	Black	1.0	6.5
C ₆	Black	1.25	6.2
C ₇	Grey	3.0	5.2
K ₁	Black	12.1	4.2
K ₂	Grey	9.0	5.1
K ₃	Black	8.5	5.6
K ₄	Yellow	4.8	6.2
K ₅	Yellow	2.25	6.6
K ₆	Black	3.0	6.8
A ₁	Black	1.5	6.5
A ₂	Green	3.1	5.8
A ₃	Yellow	1.1	6.2
A ₄	Green	4.0	6.5
A ₅	Black	0.71	6.2
A ₆	White mycelia	1.9	6.6

Mutation of isolate K₁ by N-methyl-N'-nitro-N-nitrosoguanidine (NTG). A fresh spore suspension was treated with 100 and 200 µg/ml of NTG. The survival count was taken at different intervals of time.

After 15 and 30 min of treatment with the mutagen (100 µg/ml) the survival count was 75 and 42%, respectively. After 60 min treatment at 200 µg/ml concentration 0.12% of the spores survived. The survivors were screened for morphology, colour of spores, and the nature and size of clear zones. Seven distinct colonies were selected and maintained after purification on potato dextrose agar slants (Table II).

Table II

Survival count of isolate K₁ using NTG (200 µg/ml) as mutagen

Time interval (min)	No. of surviving spores	Survival per cent
0	70×10^5	100
15	33×10^5	47.1
30	80×10^4	11.4
40	15×10^4	2.1
50	45×10^3	0.6
60	86×10^2	0.12
90	18×10^2	0.02

Carboxy methyl cellulase (CMCase) activity of mutants. The 7 probable mutants of isolate K₁ selected after treatment with NTG were grown in liquid cultured on REESE and MANDELS medium [4]. Their capacity to produce CMCase is presented in Table III. Only three mutants (KM₄, KM₅ and KM₇) showed higher CMCase activity. The remainder either lost their capacity to synthesize CMCase (KM₁, KM₂, KM₃) or showed a decreased activity (KM₆). The mutant designated KM₇ (Fig. 1) showed a three-fold increase in enzyme production (36.5 units/ml) as compared with the wild-type.

In the case of *T. viride* QM 6a the CMCase yield was increased by mutation by means of irradiating the conidia with a linear accelerator [9]. In the case of the thermotolerant *Aspergillus terreus*, UV irradiation together with ethyl-enimine was used to produce a mutant with increased cellulolytic activity [10].

The enzyme filtrate of mutant KM₇ was further analysed for β-glucosidase and Avicelase activity. The results (Table IV) were compared with the standard organism *T. viride* QM-9414. The standard strain produced the highest levels of CMCase but the mutant KM₇ produced eight times as much β-glucosidase than did the wild-type and about 67% more than *T. viride* QM-9414. Avicelase

Table III
CMCase activity of mutants of isolate K₁

Mutant	CMCase activity (units/ml)	Final pH of filtrate	Total reducing sugars in the filtrate ($\mu\text{g/ml}$)
K ₁ (parent)	12.5	4.6	75
KM ₁	nil	4.2	nil
KM ₂	nil	4.4	nil
KM ₃	nil	4.1	nil
KM ₄	15.0	4.5	102
KM ₅	18.0	4.5	nil
KM ₆	9.0	4.3	104
KM ₇	36.5	4.1	70

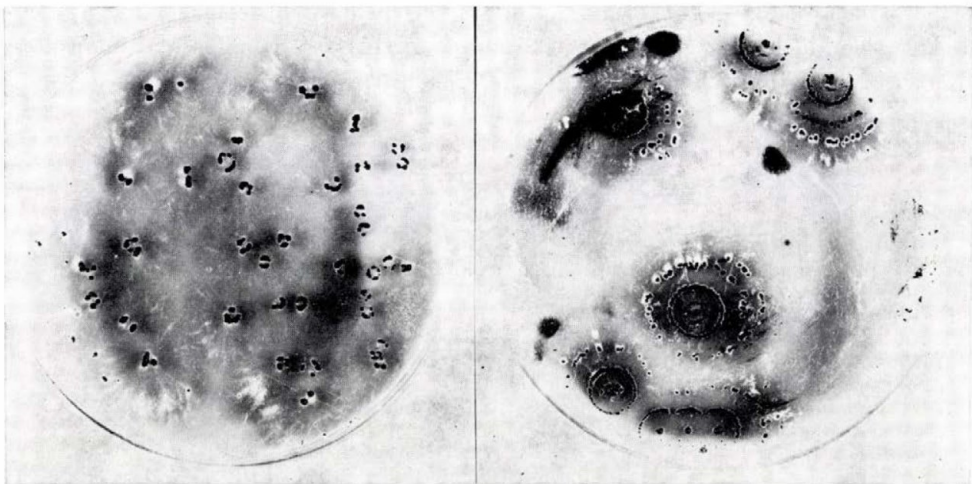


Fig. 1. Mutation of isolate K₁ with NTG. (left) K₁ wild type on RBCA medium; (right) KM mutant on RBCA medium

activity was not detected in either K₁ or KM₇, while 880 units/ml were produced by *T. viride* QM-9414. This clearly indicated that K₁ and KM₇ were pseudo-cellulolytic since they lacked the C₁ component of the cellulolytic enzyme complex and therefore could not degrade crystalline/microcrystalline cellulosic materials. True cellulolytic fungi contain all three components, i.e. C₁, C_x and β -glucosidase. These fungi which produce enzyme filtrates capable of degrading native and crystalline cellulose include *Myrothecium verrucaria* [11], *T. viride* [12], *T. koningii* [13] and *Sporotrichum pulverulentum* [14].

Table IV

CMCase β -glucosidase and avicelase activity of K_1 , KM_7 and *T. viride* QM-9414

Organism (Mutant)	CMCase activity (units/ml)	Avicelase activity (units/ml)	β -Glucosidase activity (units/ml)	Final pH of the filtrate	Total reducing sugars in the filtrate (μ g/ml)
K_1	12.1	nil	48	4.5	80
KM_7	36.2	nil	400	4.1	70
<i>T. viride</i>					
QM-9414	78.5	880	240	4.5	280

Mutant KM_7 was found to be a good source of β -glucosidase. This enzyme catalyses the last reaction in the degradation of cellulose to glucose. Beta-glucosidase plays an important role in controlling the glucose-cellobiose ratio during saccharification [15]. STERNBERG *et al.* [16] screened a large number of organisms and found that while *T. viride* was generally deficient in β -glucosidase, the black aspergilli were a rich source of this enzyme. The parent isolate K_1 was identified as belonging to genus *Phome*, which are recognized as wood decaying fungi [17, 18].

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SALMONELLA AND SHIGELLA SURVEILLANCE IN HUNGARY 1972–1976

I. SALMONELLA SURVEILLANCE

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Salmonellae other than *S. typhi*, *S. paratyphi-A*, *S. paratyphi-B* and *S. paratyphi-C*, were isolated from 35 133 persons in the bacteriological laboratories of the Public Health Stations between 1972 and 1976. The number of isolations varied between 6152 and 8165 yearly; the majority was reported in the third quarter of the year. Up to the end of 1976, salmonellae belonging to 189 different serotypes were isolated in Hungary and 35 of them were first encountered between 1972 and 1976; 66–75 serotypes occurred yearly, the 10 most frequently isolated ones comprised 72.6–82.7% of the total of isolations. *S. typhi-murium* was the most frequent in every year, the order of further types varied yearly. The number of salmonella isolations varied in different regions, the rate for 100 000 persons was highest in Veszprém, Vas and Komárom counties. The number of serotypes occurring in a given area varied between 21 and 97. Ten serotypes (*S. typhi-murium*, *S. stanley*, *S. enteritidis*, *S. panama*, *S. anatum*, *S. abony*, *S. saint-paul*, *S. bareilly*, *S. derby*, *S. thompson*) occurred in every location in each year. The most wide-spread serotype was *S. typhi-murium* followed by *S. enteritidis*. One-third of the isolations derived from epidemics and two-thirds from sporadic cases. In Vas, Komárom and Bács-Kiskun counties, however, more isolations were made from epidemics than from sporadic cases. Two-thirds of the salmonella positive persons were patients and one-third asymptomatic excreters. The rate of *S. typhi-murium* isolations from patients and asymptomatic excreters was 2.8–3.0:1, the rate was 2.0–3.0:1 in the case of *S. enteritidis*. The difference was significantly less with other serotypes. During the 5-year surveillance, 2619 epidemics including 14 501 cases were announced; 85% of the cases were confirmed bacteriologically. Detailed data are available for 1105 epidemics; 62.1% of them occurred in families and 27.8% in communities. In 58.7% of the epidemics the infection was spread by food and in 18.3% by contact routes. Seventy-five of the outbreaks spreading by contact occurred in hospitals and involved 1140 patients including more than 50% newborn or premature infants with 20 deaths. The hospital epidemics were caused by 25 different salmonella serotypes, *S. kapemba*, *S. panama* and *S. typhi-murium* being responsible for the most wide-spread outbreaks. The majority of the *S. typhi-murium* strains belonged to phage-type 4. The rate of untypable *S. typhi-murium* isolates increased yearly, reaching the incidence of phage-type 4 strains in 1976. Six out of the eight biochemical types, used for the subdivision of *S. typhi-murium* phage types, were encountered; types 1, 2 and 3 comprised the majority of isolates.

The frequent occurrence of salmonellae throughout the world and the varying geographical distribution and epidemiological significance of the serotypes have laid emphasis on the collection of relevant informations. To coordinate the reporting and evaluation of data, the World Health Organization has started an International Salmonella Surveillance Programme based on national surveillance in each participating country. In Hungary, the National Salmonella Centre operating in this Institute is in charge of the surveillance activities. The present paper gives an account of salmonellae other than *S. typhi*, *S. paratyphi-A*, *S. paratyphi-B* and *S. paratyphi-C*, isolated from human samples in the years 1972–1976.

Materials and methods

Isolation and identification of salmonellae. Human samples (mostly faeces) were examined in laboratories of the regional Public Health Stations. Salmonellae were isolated using bismuth sulphite agar, desoxycholate citrate agar and selenite brilliant green enrichment broth for primary seeding of the samples. All isolates were checked for biochemical features and were serotyped using agglutinating sera prepared and distributed by this Institute. Phage typing of *S. typhi-murium* was performed according to the Felix-Callow scheme and was supplemented by biochemical classification of the isolates into 8 biotypes on the basis of reactions in rhamnose and inositol.

Collection of data. Since 1972, the data have been reported as specified in the WHO forms. Corresponding to WHO terminology, one "isolation" means one isolation per person for a given serotype, "epidemic outbreak" means at least two cases having a connexion, even if these two cases were asymptomatic, and "single" or "sporadic" case refers to an isolation without any relationship with other cases of salmonellosis.

Results and discussion

Yearly incidence of salmonella isolations. In the years 1972-1976, salmonellae were isolated from a total of 35 133 persons (Table I). The number of isolations decreased in 1973 and 1974, but in the next year increased considerably and in 1976 reached a peak highest than ever in this country. The number of isolations was usually the lowest in the first quarter of the year and the highest in the third quarter. In 1972 and 1975, however, the peak of isolations occurred in the second quarter.

Incidence of serotypes isolated in Hungary. Table II shows the serotypes encountered in the years 1972-1976. Altogether 125 different serotypes were isolated, more than in any of the preceding five-year periods. (Between 1930 and 1976, 189 kinds of serotypes were shown in Hungary.) In the years 1972-

Table I
Isolations of Salmonella from man in Hungary,
1972-1976*

Year	January-March	April-June	July-September	October-December	Total
1972	1024	2644	2152	1162	6982
1973	791	1786	2667	1340	6584
1974	869	1805	2184	1294	6152
1975	975	2342	2264	1669	7250
1976	916	2349	2779	2121	8165
Total	4575	10926	12046	7586	35133

* The data represent one isolation per person for a given serotype

Table II

Salmonella serotypes isolated in Hungary, 1972-1976
Number of serotypes, 125

<i>S. abony</i>	<i>S. fyris</i> ²	<i>S. muenster</i>
<i>S. abortus-bovis</i>	<i>S. gallinarum</i>	<i>S. nagoya</i>
<i>S. abortus-equi</i>	<i>S. give</i>	<i>S. newington</i>
<i>S. africana</i> ¹	<i>S. gloucester</i> ¹	<i>S. newport</i>
<i>S. agona</i>	<i>S. gnesta</i> ²	<i>S. nyborg</i> ²
<i>S. albany</i> ¹	<i>S. goeteborg</i> ²	<i>S. ohio</i>
<i>S. amager</i>	<i>S. hadar</i> ¹	<i>S. onireke</i>
<i>S. amsterdam</i> ¹	<i>S. haifa</i>	<i>S. oranienburg</i>
<i>S. anatum</i>	<i>S. havana</i>	<i>S. orion</i>
<i>S. bareilly</i>	<i>S. heidelberg</i>	<i>S. oslo</i> ²
<i>S. binza</i>	<i>S. indiana</i>	<i>S. panama</i>
<i>S. bispesbjerg</i> ¹	<i>S. infantis</i>	<i>S. pikine</i>
<i>S. blockley</i>	<i>S. irumu</i>	<i>S. poona</i> ²
<i>S. bonn</i> ²	<i>S. isangi</i>	<i>S. potsdam</i>
<i>S. bovis-morbificans</i>	<i>S. java</i>	<i>S. reading</i>
<i>S. bradford</i>	<i>S. jerusalem</i> ²	<i>S. richmond</i>
<i>S. braenderup</i>	<i>S. kaapstad</i>	<i>S. rissen</i> ¹
<i>S. brandenburg</i>	<i>S. kapemba</i>	<i>S. saint-paul</i>
<i>S. bredeney</i>	<i>S. kentucky</i>	<i>S. san-diego</i>
<i>S. butantan</i>	<i>S. kilwa</i>	<i>S. san-juan</i> ²
<i>S. cairo</i>	<i>S. kimuenza</i>	<i>S. schleissheim</i>
<i>S. calabar</i>	<i>S. kinshasa</i> ²	<i>S. schwarzengrund</i>
<i>S. chailey</i> ¹	<i>S. kivu</i> ²	<i>S. sendai</i> ¹
<i>S. champaign</i> ²	<i>S. kottbus</i>	<i>S. senftenberg</i>
<i>S. chester</i>	<i>S. lagos</i>	<i>S. shubra</i> ²
<i>S. chincol</i>	<i>S. lexington</i>	<i>S. sinstorff</i> ¹
<i>S. cholerae-suis</i>	<i>S. lille</i>	<i>S. solt</i>
<i>S. coleypark</i>	<i>S. limete</i>	<i>S. stanley</i>
<i>S. colindale</i> ²	<i>S. livingstone</i>	<i>S. stanleyville</i>
<i>S. concord</i>	<i>S. loma-linda</i> ²	<i>S. stratford</i> ²
<i>S. corvallis</i>	<i>S. lomita</i>	<i>S. szentes</i>
<i>S. cubana</i>	<i>S. london</i>	<i>S. takoradi</i>
<i>S. daytona</i> ²	<i>S. manchester</i>	<i>S. tennessee</i>
<i>S. derby</i>	<i>S. manhattan</i>	<i>S. thompson</i>
<i>S. duisburg</i> ²	<i>S. manila</i>	<i>S. typhi-murium</i>
<i>S. durban</i> ²	<i>S. meleagridis</i>	<i>S. victoria</i>
<i>S. eastbourne</i>	<i>S. memphis</i> ²	<i>S. westhampton</i>
<i>S. edinburg</i> ²	<i>S. minnesota</i>	<i>S. wien</i> ¹
<i>S. enteritidis</i>	<i>S. mishmar-haemek</i> ²	<i>S. wippra</i> ¹
<i>S. eppendorf</i>	<i>S. mokola</i>	<i>S. worthington</i>
<i>S. essen</i>	<i>S. montevideo</i>	<i>S. zanzibar</i>
<i>S. farmsen</i> ¹	<i>S. muenchen</i>	

¹ First isolation in Hungary

² First isolation in Hungary; isolated only in the year of appearance

1976, 35 *Salmonella* serotypes were isolated which had not been found previously in this country. One-third of these serotypes occurred in 1972; most of them were "exotic" types isolated from persons arriving from abroad. Twenty-two of the serotypes isolated for the first time occurred only in the year of isolation and were cultured mostly from asymptomatic excreters.

Table III

The 10 most frequently reported *Salmonella* serotypes in Hungary, 1972-1976

Serotype	1972 (6982 isolations)		1973 (6584 isolations)		1974 (6152 isolations)		1975 (7250 isolations)		1976 (8165 isolations)	
	Rank	%	Rank	%	Rank	%	Rank	%	Rank	%
<i>S. typhi-murium</i>	1	26.8	1	32.2	1	37.7	1	35.1	1	26.6
<i>S. stanley</i>	2	10.6	9	2.6	5	4.5	7	3.8		
<i>S. kapemba</i>	3	6.2								
<i>S. enteritidis</i>	4	5.8	2	18.6	2	10.8	4	6.8	2	7.4
<i>S. panama</i>	5	5.5	6	4.1	3	6.5	6	4.8	3	6.5
<i>S. give</i>	6	5.5	4	5.4						
<i>S. cubana</i>	7	5.5								
<i>S. anatum</i>	8	5.1	7	3.5	6	3.7				
<i>S. manhattan</i>	9	4.4					9	3.2		
<i>S. abony</i>	10	3.8	5	4.9	10	2.5				
<i>S. saint-paul</i>			3	6.0	4	4.6	5	4.9	4	6.3
<i>S. bareilly</i>			8	3.0						
<i>S. derby</i>			10	2.4	7	3.2	2	8.4	5	5.4
<i>S. thompson</i>					8	2.8	10	2.5	6	4.9
<i>S. infantis</i>					9	2.8	8	3.5	9	3.6
<i>S. agona</i>							3	6.9	7	4.8
<i>S. london</i>									8	3.7
<i>S. brandenburg</i>									10	3.4
Total	1-10	79.2	1-10	82.7	1-10	79.1	1-10	79.7	1-10	72.6
	11-15	9.7	11-15	8.6	11-15	9.9	11-15	9.7	11-15	11.7
	16-71	9.8	16-71	7.7	16-75	9.5	16-66	10.0	16-72	14.2
Not typed		1.3		1.0		1.5		0.6		1.5

Table III shows the order of frequency for the 10 commonest serotypes in each year and their ratio in per cent, compared to the total percentage of the remaining serotypes. The number of different kinds of serotypes isolated in one year was between 66 and 75. The yearly incidence of the 10 most frequent serotypes was 72.6-82.7% of all isolations. *S. typhi-murium*, *S. enteritidis* and *S. panama* ranked in each year among the 10 commonest serotypes, whereas *S. stanley*, *S. saint-paul* and *S. derby* fell in this category in four years of the five-year period. *S. typhi-murium* was the most frequent in each year (26.6-37.7%). The rank of other serotypes varied yearly. The number of *S. kapemba*,

Table IV

Geographic distribution of salmonella isolations in Hungary, 1972-1976

Location (county or Budapest)	Salmonella isolations, 1972-1976		
	No.	Per cent of total	Yearly average for 100 000 inhabitants
Baranya	1112	3.2	51.5
Bács-Kiskun	568	1.6	20.2
Békés	632	1.8	29.2
Borsod-Abaúj-Zemplén	1103	3.1	28.2
Csongrád	1843	5.3	81.5
Fejér	2044	5.8	101.2
Győr-Sopron	1221	3.5	58.5
Hajdú-Bihar	1325	3.8	49.6
Heves	854	2.4	50.1
Komárom	1900	5.4	122.2
Nógrád	706	2.0	60.3
Pest	2178	6.2	47.0
Somogy	1272	3.6	70.4
Szabolcs-Szatmár	1065	3.0	37.8
Szolnok	1564	4.5	75.5
Tolna	536	1.5	42.5
Vas	1790	5.1	128.3
Veszprém	3492	9.9	165.8
Zala	1187	3.4	90.8
Budapest	8741	24.9	85.2
Total	35133	100.0	67.2

S. cubana and *S. manhattan* isolations decreased from 1973 onward. From 1974, *S. agona*, *S. derby*, *S. infantis*, *S. london* and *S. thompson* became more prominent.

Regional distribution of salmonella isolations. As shown in Table IV, the number of salmonella isolations varied with the geographic areas of the country. The average ratio of isolation for 100 000 inhabitants was 67.2, and in this respect, too, there was a significant difference between localities. The number of serotypes reported between 1972 and 1976 in the different areas varied between 21 and 97 (Table V).

Table V
Geographic distribution of the most frequently

Serotype	Baranya	Bács-Kiskun	Békés	Borsod-Abaúj-Zemplén	Csongrád	Fejér	Győr-Sopron	Hajdú-Bihar	Heves
<i>S. typhi-murium</i>	604	274	257	436	451	888	285	606	324
<i>S. stanley</i>	22	140	2	33	57	157	55	10	14
<i>S. kapemba</i>	—	1	—	—	7	105	130	2	2
<i>S. enteritidis</i>	106	13	72	69	95	257	75	55	151
<i>S. panama</i>	30	6	38	32	53	48	85	53	19
<i>S. give</i>	6	—	3	1	3	52	11	11	4
<i>S. cubana</i>	—	—	3	14	21	11	12	13	15
<i>S. anatum</i>	52	7	3	29	148	47	20	17	20
<i>S. manhattan</i>	4	1	—	12	6	24	1	42	15
<i>S. abony</i>	38	26	31	30	151	58	7	38	46
<i>S. saint-paul</i>	62	11	35	83	125	41	13	78	24
<i>S. bareilly</i>	28	4	22	82	52	64	28	34	12
<i>S. derby</i>	21	3	20	14	77	67	58	83	45
<i>S. thompson</i>	5	39	50	15	47	54	20	14	32
<i>S. infantis</i>	49	4	—	27	26	52	44	13	16
<i>S. agona</i>	2	8	—	18	54	36	55	13	3
<i>S. london</i>	27	1	—	—	260	4	5	—	5
<i>S. brandenburg</i>	8	—	—	69	10	3	62	22	27
Total, serotypes listed	1064	538	536	964	1643	1968	966	1104	774
Total, other serotypes	45	22	75	113	162	70	136	185	79
Total, not typed	3	8	21	26	38	6	119	36	1
Total, all isolations	1112	568	632	1103	1843	2044	1221	1325	854
Number of serotypes	31	21	29	27	40	38	34	36	38

Salmonellae falling to the first 10 serotypes in the yearly rank order, were represented by 18 serotypes during the five-year survey period. Ten of these 18 serotypes occurred in all regions, *S. typhi-murium* being uniformly the most frequent in every year and every location. Next in order were *S. enteritidis*, *S. panama*, *S. stanley*, *S. saint-paul*, *S. derby*, *S. anatum*, *S. abony*, *S. thompson* and *S. bareilly*, but their incidence varied greatly from region to region.

reported *Salmonella* serotypes, 1972-1976

Komárom	Nógrád	Pest	Somogy	Szabolcs-Szatmár	Szolnok	Tolna	Vas	Veszprém	Zala	Budapest	Total
869	264	658	536	447	505	242	454	1015	283	1638	11036
27	21	67	37	1	25	54	63	265	165	423	1638
10	36	47	19	—	4	4	4	517	33	109	1030
303	68	116	67	20	234	58	240	245	136	1009	3389
161	17	132	77	40	42	39	95	246	41	684	1938
12	4	59	3	2	58	—	24	44	257	477	1031
14	3	55	1	14	3	—	1	42	14	355	591
16	17	74	111	28	16	5	11	58	7	433	1119
34	2	81	2	2	11	1	3	1	2	471	715
16	24	123	36	13	83	2	23	11	54	190	1000
97	57	179	37	47	143	10	38	62	19	433	1594
19	21	50	14	65	47	3	47	37	19	78	726
33	24	91	132	17	58	22	251	7	25	489	1537
103	16	54	41	5	29	2	26	195	44	198	989
53	7	98	70	9	21	—	63	62	5	344	963
39	—	72	42	43	14	—	179	15	34	524	1151
1	—	14	5	—	10	3	4	—	—	39	378
19	2	17	3	11	27	4	118	—	7	79	488
1826	583	1987	1233	764	1330	449	1644	2822	1145	7973	31313
67	121	183	38	294	185	84	143	642	34	720	3398
7	2	8	1	6	51	3	3	28	8	47	422
1900	706	2178	1272	1064	1566	536	1790	3492	1187	8740	35133
38	27	48	26	26	35	28	34	37	28	97	127

Certain serotypes caused transient or prolonged epidemiological problems in some districts, e.g. *S. brandenburg* in counties Vas, Borsod-Abaúj-Zemplén and Győr-Sopron, *S. kapemba* in Fejér, Győr-Sopron and Veszprém, *S. london* (inositol negative biovariant) in Csongrád, *S. agona* in Vas.

Of the less frequently isolated serotypes 53 occurred only in one given district where, however, some of them were fairly often encountered (e.g.

Table VI
Geographic distribution of salmonella isolations from epidemics and sporadic cases, 1972-1976

Location (county or Budapest)	Salmonella isolations					Epidemics	
	Total no.	epidemics		sporadic cases		No.	Per cent for total number of epidemics
		No.	Per cent for location	No.	Per cent for location		
Baranya	1112	513	46.1	599	53.9	48	1.8
Bács-Kiskun	568	304	53.5	264	46.5	38	1.5
Békés	632	151	23.9	481	76.1	14	0.5
Borsod-Abaúj-Zemplén	1103	302	27.4	801	72.6	60	2.3
Csongrád	1843	613	33.3	1230	66.7	136	5.2
Fejér	2044	563	27.5	1481	72.5	77	2.9
Győr-Sopron	1221	475	38.9	746	61.1	105	4.0
Hajdú-Bihar	1325	429	32.4	896	67.6	134	5.1
Heves	854	406	47.5	448	52.5	106	4.1
Komárom	1900	1125	59.2	775	40.8	362	13.8
Nógrád	706	225	31.9	481	68.1	73	2.8
Pest	2178	512	23.5	1666	76.5	120	4.6
Somogy	1272	535	42.1	737	57.9	162	6.2
Szabolcs-Szatmár	1064	278	26.1	786	73.9	53	2.0
Szolnok	1566	685	43.7	881	56.3	154	5.9
Tolna	536	177	33.0	359	67.0	46	1.8
Vas	1790	1075	60.1	715	39.9	147	5.6
Veszprém	3492	1634	46.8	1858	53.2	223	8.5
Zala	1187	547	46.1	640	53.9	98	3.7
Budapest	8740	1779	20.4	6961	79.6	463	17.7
Total	35133	12328	35.1	22805	64.9	2619	100.0

S. essen, *S. lagos* and *S. victoria* in Veszprém, *S. irumu* in Szabolcs-Szatmár, *S. butantan* in Szolnok).

Salmonella isolations from epidemic outbreaks and from single cases. In the years 1972-1976, about one third of the isolations were made from epidemic outbreaks, the rest (64.9%) from sporadic cases (Table VI). A similar distribution was characteristic of the yearly isolations. The ratio of epidemic and sporadic incidence varied with the localities; in counties Vas, Komárom and Bács-Kiskun the majority of salmonellae originated from epidemics. Most of the outbreaks occurred in Budapest (17.7%) and in counties Komárom

Table VII

Percentage distribution of *Salmonella* serotypes isolated from epidemics and sporadic cases, 1972-1976

	1972		1973		1974		1975		1976	
	Epidemics	Sporadic cases	Epidemics	Sporadic cases	Epidemics	Sporadic cases	Epidemics	Sporadic cases	Epidemics	Sporadic cases
<i>S. typhi-murium</i>	43.2	56.8	42.1	57.9	41.7	58.3	44.4	55.6	31.9	68.1
<i>S. stanley</i>	60.9	39.1	20.7	79.3	39.1	60.9	19.2	80.8		
<i>S. kapemba</i>	64.4	35.6								
<i>S. enteritidis</i>	17.8	82.2	66.3	33.7	62.2	37.8	47.3	52.7	39.9	60.1
<i>S. panama</i>	27.6	72.4	26.5	73.5	43.5	56.5	18.6	81.4	33.6	66.4
<i>S. give</i>	16.7	83.3	16.7	83.3						
<i>S. cubana</i>	12.5	87.5								
<i>S. anatum</i>	28.3	71.7	40.6	59.4	15.7	84.3				
<i>S. manhattan</i>	18.7	81.3					22.4	77.6		
<i>S. abony</i>	13.1	86.9	38.5	61.5	23.2	76.8			38.9	61.1
<i>S. bareilly</i>			27.3	72.7						
<i>S. derby</i>			45.9	54.1	22.8	77.2	43.3	56.7	15.7	84.3
<i>S. thompson</i>					24.7	75.3	50.0	50.0	60.7	39.3
<i>S. infantis</i>					14.8	85.2	21.5	78.5	17.2	82.8
<i>S. agona</i>							30.4	69.6	33.9	66.1
<i>S. london</i>									49.8	50.2
<i>S. brandenburg</i>									28.7	71.3
Total, serotypes listed	36.6	64.4	43.5	56.5	39.7	60.3	37.2	62.8	34.4	65.6
Total, other serotypes	22.8	77.2	22.8	77.2	16.2	83.8	25.9	74.1	29.9	70.1
Total, all serotypes	33.4	66.6	39.7	60.3	34.7	65.3	35.3	64.7	32.9	67.1

Table VIII

Geographic distribution of salmonella isolations from patients and symptom-free excretors, 1972-1976

Area (county or Budapest)	Salmonella isolation				
	Total no.	Patients		Asymptomatic excretors	
		No.	%	No.	%
Baranya	1056	638	60.4	418	39.6
Bács-Kiskun	410	340	82.9	70	17.1
Békés	567	346	61.0	221	39.0
Borsod-Abaúj-Zemplén	948	626	66.0	322	34.0
Csongrád	1608	752	46.8	856	53.2
Fejér	1484	929	62.6	555	37.4
Győr-Sopron	1073	792	73.8	281	26.2
Hajdú-Bihar	1190	801	67.3	389	32.7
Heves	670	492	73.4	178	26.6
Komárom	1522	1197	78.6	325	21.4
Nógrád	600	384	64.0	216	36.0
Pest	1759	1015	57.7	744	42.3
Somogy	1063	867	81.6	196	18.4
Szabolcs-Szatmár	934	726	77.7	208	32.3
Szolnok	1382	934	67.6	448	32.4
Tolna	450	370	82.2	80	17.8
Vas	1606	1298	80.8	308	19.2
Veszprém	2560	1308	51.1	1252	48.9
Zala	769	450	58.5	319	41.5
Budapest	6500	3963	61.0	2537	39.0
Total	28151	18228	64.8	9923	35.2

(13.8%) and Veszprém (8.5%). The average of bacteriologically confirmed cases of salmonellosis for one epidemic ranged between 3.1 and 10.8 in different localities, the country-wide average being 4.7.

Epidemic and sporadic occurrence differed according to the serotypes implicated (Table VII). In 1972-1975 more than 40% of *S. typhi-murium* isolates came from epidemic outbreaks; in 1976 this ratio decreased to 30%. In 1972 *S. enteritidis* was isolated mostly from single cases, but in the following two years mainly from outbreaks; from 1975 onward, sporadic isolations have again assumed prominence. Of other serotypes, in 1972 *S. stanley* and *S. kapemba*, in 1976 *S. thompson* were rather frequently isolated from outbreaks.

Table IX

Percentage distribution of *Salmonella* serotypes isolated from patients and asymptomatic excretors, 1973-1976

Serotype	1973			1974			1975			1976		
	Patients	Excretors	Patients/ excretors	Patients	Excretors	Patients/ excretors	Patients	Excretors	Patients/ excretors	Patients	Excretors	Patients/ excretors
<i>S. typhi-murium</i>	75.2	24.8	3.0: 1	73.8	26.2	2.8: 1	75.1	24.9	3.0: 1	74.4	25.6	2.9: 1
<i>S. enteritidis</i>	75.2	24.8	3.0: 1	71.2	28.8	2.5: 1	70.9	29.1	2.4: 1	67.1	32.9	2.0: 1
<i>S. saint-paul</i>	61.2	38.8	1.6: 1	59.6	40.4	1.5: 1	60.5	39.5	1.5: 1	52.8	47.2	1.1: 1
<i>S. give</i>	51.6	48.4	1.1: 1									
<i>S. abony</i>	57.5	42.5	1.4: 1	69.7	30.3	2.3: 1						
<i>S. panama</i>	58.8	41.2	1.4: 1	47.5	52.5	0.9: 1	61.7	38.3	1.6: 1	60.9	39.1	1.6: 1
<i>S. anatum</i>	37.2	62.8	0.6: 1	55.5	44.5	1.3: 1						
<i>S. bareilly</i>	52.5	47.5	1.1: 1									
<i>S. stanley</i>	65.1	34.9	1.9: 1	62.0	38.0	1.6: 1	66.3	33.7	2.0: 1			
<i>S. derby</i>	50.3	49.7	1.0: 1	59.9	40.1	1.5: 1	64.5	35.5	1.8: 1	60.5	39.5	1.5: 1
<i>S. thompson</i>				51.7	42.9	1.3: 1	58.3	41.7	1.4: 1	57.7	42.3	1.4: 1
<i>S. infantis</i>				60.4	39.6	1.5: 1	58.6	41.4	1.4: 1	57.9	42.1	1.4: 1
<i>S. agona</i>							67.1	32.9	2.0: 1	60.5	39.5	1.5: 1
<i>S. manhattan</i>							56.9	43.1	1.3: 1			
<i>S. london</i>										32.0	68.0	0.5: 1
<i>S. brandenburg</i>										59.6	40.4	1.5: 1
Total, serotypes listed	67.3	32.7	2.1: 1	67.2	32.8	2.1: 1	68.9	31.1	2.2: 1	63.8	36.2	1.8: 1
Total, other serotypes	56.8	43.2	1.3: 1	58.4	41.6	1.4: 1	59.7	40.3	1.5: 1	54.5	45.5	1.2: 1
Total, all serotypes	65.5	34.5	1.9: 1	65.4	34.6	1.9: 1	67.1	32.9	2.1: 1	61.5	38.5	1.6: 1

Table X
Number of salmonellosis outbreaks and cases, 1972-1976

Year	No. of outbreaks	No. of cases belonging to the outbreaks			
		Confirmed by isolation		Diagnosed on epidemiological grounds	Total no.
		Patients	Excreters		
1972	482	2334		381	2715
1973	481	1695	921	479	3095
1974	506	1358	776	387	2521
1975	486	1687	874	620	3181
1976	664	1420	1263	306	2989
Total	2619	12328		2173	14501

Salmonella isolations from patients and asymptomatic excreters. Nation-wide data have been collected since 1973 (Table VIII). Out of the 28 151 salmonella isolations in 1973-1976, 64.8% belonged to patients, 35.2% to asymptomatic excreters. Yearly data showed little difference from this distribution and in all regions, except Csongrád, the majority of isolations represented persons with clinical symptoms of salmonellosis. The ratio of patients was higher than the average (more than 80%) in Bács-Kiskun, Somogy, Tolna and Vas.

There was a difference between serotypes as to the ratio of isolations from patients and asymptomatic excreters (Table IX). *S. typhi-murium* was isolated in about 75% from patients in each year (patient/excreter ratio 2.8-3.0: 1).

Table XI
Outbreaks of salmonellosis according to mode of infection, 1972-1976

Mode of infection	Family outbreaks, no. of		Community outbreaks, no. of		Other outbreaks, no. of		Total			
	out-breaks	cases	out-breaks	cases	out-breaks	cases	outbreaks		cases	
							No.	%	No.	%
Food	490	1771	80	2896	79	3574	649	58.7	8241	72.6
Water	—	—	1	28	2	39	3	0.3	67	0.6
Contact route	17	65	180	1737	5	30	202	18.3	1832	16.1
Unknown	179	474	46	529	26	204	251	22.7	1207	10.7
Total	686	2310	307	5190	112	3847	1105	100.0	11347	100.0
Per cent	62.1	20.4	27.8	45.7	10.1	33.9	100.0		100.0	

Table XII

Outbreaks in communities spreading by contact route, 1972-1976

Community	No. of outbreaks	No. of cases	Cases per outbreak
Day nurseries	37	229	6.2
Kindergartens	47	240	5.1
Children and infants homes	6	71	11.8
Obstetric wards	3	48	16.0
Newborn wards	7	432	61.7
Premature wards	7	147	21.0
Paediatric wards	30	238	7.9
Other wards	28	275	9.8
Adults communities	15	57	3.8
Total	180	1737	9.7

S. enteritidis was also considerably more prevalent in patients (2.0-3.0:1). Other serotypes occurred more frequently in patients, but the difference in patient/excreter ratio was less marked. Exceptionally, the number of asymptomatic excreters was higher than that of patients (*S. anatum* in 1973, *S. panama* in 1974, *S. london* in 1976).

Outbreaks of salmonellosis. In the period between 1972 and 1976, 2619 outbreaks involving 14 501 cases were reported. Of the cases 12 328 (85.0%) were confirmed by isolation of the causative agent, 2173 (15.0%) were diagnosed on epidemiological grounds only (Table X).

As shown in Table XI, more detailed data were available for 1105 outbreaks (42.2% of all outbreaks) with 11 347 cases (78.2% of all epidemic cases). It is seen that the majority of outbreaks occurred in single families, whereas community outbreaks involved almost half of the total number of cases.

Seventy-five (41.7%) out of 180 community outbreaks spreading by the contact route, including 1140 (65.6%) out of 1737 cases occurred in hospital wards (Table XII). More than half of the patients were newborn or premature infants. The number of cases per outbreak was very high in infant wards and high in premature wards and in wards caring for both infants and young children.

Hospital outbreaks were associated with 25 different serotypes, *S. kapemba*, *S. panama* and *S. typhi-murium* being the most frequently incriminated (Table XIII). In these outbreaks 20 patients, mainly of the youngest and oldest age groups, died.

Table XIII

Distribution according to serotypes of hospital outbreaks spreading by contact route, 1972-1976

Serotype	No. of outbreaks	No. of cases	No. of deaths
<i>S. agona</i>	4	18	
<i>S. anatum</i>	2	49	3
<i>S. brandenburg</i>	2	7	
<i>S. bredeney</i>	3	51	
<i>S. butantan</i>	1	2	
<i>S. chailey</i>	1	3	
<i>S. cubana</i>	3	14	
<i>S. derby</i>	2	6	
<i>S. eastbourne</i>	1	7	
<i>S. enteritidis</i>	6	42	1
<i>S. essen</i>	1	4	
<i>S. give</i>	2	24	
<i>S. heidelberg</i>	1	8	
<i>S. infantis</i>	2	40	
<i>S. java</i>	2	55	
<i>S. kapemba</i>	6	315	3
<i>S. kottbus</i>	1	2	
<i>S. london</i>	1	8	
<i>S. manhattan</i>	3	56	2
<i>S. oslo</i>	1	3	
<i>S. panama</i>	6	173	
<i>S. saint-paul</i>	8	54	6
<i>S. stanley</i>	2	19	
<i>S. thompson</i>	1	18	
<i>S. typhi-murium</i>	13	162	5
Total	75	1140	20

Phage type distribution of S. typhi-murium. The majority of *S. typhi-murium* strains of human origin belonged to phage type 4 in the years 1972-1976, but the frequency of this phage type decreased gradually from 1974. The incidence of untypable strains was 7.2% in 1972; their occurrence increased gradually and in 1976 they were found in 36.9%. Other frequent phage types traced were, in 1972, 1a var 1; in 1973, 1a, 2b and 2b-5; in 1974 and 1975, 2b (Table XIV). Division of the frequent phage types according to biotypes

Table XIV

Phage type distribution of S. typhi-murium strains isolated in Hungary, 1972-1976

Phage type	1972		1973		1974		1975		1976	
	No.	%	No.	%	No.	%	No.	%	No.	%
1	38	2.1	79	4.1	21	1.1	63	3.1	18	1.0
1a	98	5.6	187	9.7	68	3.7	49	2.3	75	4.1
1a var. 1	279	16.0	71	3.7	64	3.6	61	2.9	75	4.1
1a var. 1-2	2	0.1								
1b	1	<0.1					3	0.2	4	0.2
2	4	0.3	2	0.1	3	0.3	3	0.2	12	0.7
2a			2	0.1	2	0.1	3	0.2		
2b	60	3.5	133	6.9	118	6.8	107	5.4	83	4.6
2c	13	0.7	22	1.1	11	0.6	17	0.9	3	0.2
2d							24	1.2	5	0.3
3a	7	0.4	4	0.2					1	0.1
4	909	52.1	860	44.7	1017	57.7	992	49.9	675	37.0
5	53	3.0	49	2.6	38	2.2	53	2.6	63	3.5
2b-5	63	3.7	100	5.2	63	3.4	46	2.4	48	2.6
Unclassified	92	5.2	153	8.0	95	5.2	31	1.6	87	4.7
Untypable	126	7.2	261	13.6	272	15.3	540	27.1	674	36.9
Total	1745	100.0	1923	100.0	1772	100.0	1992	100.0	1823	100.0

allows a better differentiation for epidemiological purposes. Differentiation of the isolates according to biochemical reactions is presented in Table XV (overleaf).

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Table XV

Frequency of biochemically subdivided S. typhi-murium phage types in Hungary, 1972-1976

Phage type	Biochemical type*													
	1		2		3		4		5		6		Total	
	No.	%	No.	%	No.	%	No.	%	No.	%	No.	%	No.	%
1	44	20.1	77	35.2	75	34.2			22	10.0	1	0.5	219	100.0
1a	74	15.5	325	68.1	78	16.4							477	100.0
1a var 1	58	10.5	120	21.8	123	22.4	2	0.4	246	44.7	1	0.2	550	100.0
1a var 1-2			2	100.0									2	100.0
1b			2	25.0	6	75.0							8	100.0
2	3	12.5	15	62.5	5	20.8			1	4.2			24	100.0
2a			2	28.6	2	28.6			3	42.8			7	100.0
2b	80	16.0	283	56.5	135	26.9			3	0.6			501	100.0
2c	6	9.1	55	83.3	5	7.6							66	100.0
2d	1	3.4	24	82.8	4	13.8							29	100.0
3a			8	66.6	2	16.7			2	16.7			12	100.0
4	2580	57.9	450	10.1	1416	31.8			7	0.2			4453	100.0
5	3	1.2	202	78.9	51	19.9							256	100.0
2b-5	10	3.1	277	86.6	32	10.0			1	0.3			320	100.0
Unclassified	138	30.1	251	54.8	60	13.1			9	2.0			458	100.0
Untypable	92	4.9	653	34.9	1117	59.6			11	0.6			1873	100.0
Total	3089	33.3	2746	29.6	3111	33.6	2	<0.1	305	3.3	2	<0.1	9255	100.0

* Biochemical type (rhamnose/inositol): 1 +/+²⁻⁵; 2 +/-; 3 +/+; 4 -/+²⁻⁵; 5 -/-; 6 +⁸⁻¹⁰/+; 7 +⁵⁻⁸/-; 8 +⁵⁻⁸/+. Biotypes 6 and 8 were not isolated in 1972-1976

SALMONELLA AND SHIGELLA SURVEILLANCE IN HUNGARY, 1972-1976

II. SHIGELLA SURVEILLANCE

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Shigellae were isolated from 32 399 persons in the bacteriological laboratories of the Public Health Stations between 1972-1976. The number of isolations ranged between 5325 and 8237 yearly. As *S. dysenteriae* and *S. boydii* serotypes occurred only in about 1.5% of all isolations, the epidemiological situation was determined by the incidence of *S. flexneri* and *S. sonnei*. Except in 1973, *S. sonnei* constituted the majority in every year. *S. sonnei* predominance, observed first in the western regions of the country, showed a gradual eastward spread and became characteristic of all localities in 1975-1976. The July-September peak of shigella isolations was associated with *S. sonnei*, as *S. flexneri* was distributed practically evenly in every season. The incidence of shigella isolations per 100 000 inhabitants was the lowest in the middle and the highest in the northern parts of the country. Sporadic cases were somewhat more frequent than epidemic outbreaks. Shigellae were isolated in 64.1% from patients and in 35.9% from asymptomatic excretors. The patient : excretor ratio was higher for *S. sonnei* (70.2 : 29.8) than for *S. flexneri* (54.6 : 45.4). In the survey period, 14 692 isolations were made in the course of 3278 epidemics; out of these, 11 171 cases were involved in 574 extensive outbreaks. *S. sonnei* was responsible mainly for school and nursery outbreaks, whereas *S. flexneri* dysentery predominated in mental wards and in homes for the aged and for mentally retarded children. The prevalent types of *S. flexneri* were (serological/phage type): 2a/69, 3a/5, 3a/19, 4a/81, 4a/89d and 6/85. The prevalent epidemiological subunits of *S. sonnei* were (phage/colicin type): 2/0, 2/6, 2/12, 3/12, 6/0, 7/0, 65/0, 65/6 and 65/12.

Joined to the Salmonella Surveillance Programme, the Hungarian National Shigella Centre operating in this Institute, began to collect detailed data in 1972. The aim of the Programme is to survey the country-wide epidemiological situation of shigellosis and to study the features of dysentery caused by different shigellae. In the present paper we report on the first five-year period of surveillance activities.

Materials and methods

Isolation and identification of shigellae. Human faecal samples were examined in laboratories of the regional Public Health Stations. Shigellae were isolated using desoxycholate citrate agar for primary seeding of faeces. All isolates were examined for biochemical characteristics and were serotyped using agglutinating sera prepared and distributed by this Institute. Phage typing of *S. flexneri* was performed in the Department of Phage Typing of the Institute with a set of typing phages described by LÁSZLÓ *et al.* [1]. *S. sonnei* was phage typed by the method of HAMMARSTRÖM [2] as modified by KALLINGS [3]. Colicin typing of *S. sonnei* was carried out according to ABBOTT and SHANNON [4].

Collection of data was performed using the WHO terminology: one "isolation" means one isolation per person for a given serotype, epidemic outbreak means at least two cases having a connexion even if these two cases were asymptomatic, and "sporadic" case refers to an isolation without any relationship to other cases of shigellosis.

1981

Results and discussion

Yearly incidence of shigella isolations. In the years 1972–1976, shigellae were isolated from a total of 32 399 persons (Table I). The number of isolations varied from year to year: as compared to 1972, it decreased in 1973 and 1974, became higher in 1975, and decreased again in 1976.

Incidence of serotypes and epidemiological subunits of shigellae. All groups of shigellae were encountered in every year of the surveillance period. As *S. dysenteriae* and *S. boydii* serotypes were shown in each year in less than 1.9%

Table I
*Species and serotype distribution of shigella isolations in Hungary, 1972–1976**

Species and serotype	No. of isolates, year						Total	
	1972	1973	1974	1975	1976			
							No.	%
<i>S. dysenteriae</i> 2	53	63	30	41	42	229	69.0	
other and not typed	32	19	29	12	11	103	31.0	
total	85 (1.2%)	82 (1.3%)	59 (1.1%)	53 (0.06%)	53 (1.0%)	332 (1.0%)	100.0	
<i>S. flexneri</i> 1a	14	15	8	5	11	53	0.4	
1b	88	83	25	30	29	255	1.9	
1 not typed	27	—	—	—	—	27	0.2	
2a	456	487	480	398	444	2265	17.2	
2b	15	19	10	15	27	86	0.7	
2 not typed	1	—	5	1	4	11	0.1	
3a	902	879	599	549	507	3436	26.1	
3b	18	8	7	6	3	42	0.3	
3c	39	12	19	27	3	100	0.8	
3 not typed	125	161	138	129	94	647	4.9	
4a	439	351	319	322	287	1718	13.1	
4b	26	6	2	5	6	45	0.3	
4 not typed	2	—	—	—	3	5	0.1	
5	4	6	6	3	1	20	0.2	
6	140	133	99	99	160	631	4.8	
X	213	155	155	162	133	818	6.2	
Y	12	18	15	20	22	87	0.7	
not typed	890	843	656	379	119	2887	22.0	
total	3411 (47.6%)	3176 (51.5%)	2543 (46.2%)	2150 (26.1%)	1853 (34.8%)	13 133 (40.5%)	100.0	
<i>S. boydii</i>	24 (0.3%)	38 (0.6%)	34 (0.6%)	40 (0.5%)	20 (0.4%)	156 (0.5%)		
<i>S. sonnei</i>	3645 (50.9%)	2869 (46.6%)	2871 (52.1%)	5994 (72.8%)	3399 (63.8%)	18 778 (58.0%)		
All shigellae	7165 (100.0%)	6165 (100.0%)	5507 (100.0%)	8237 (100.0%)	5325 (100.0%)	32 399 (100.0%)		

* The data represent one isolation per person for a given species or serotype

Table II

Phage type distribution of frequent *S. flexneri* serotypes in percentage of strains isolated in Hungary, 1972-1976

Serotype		1972	1973	1974	1975	1976	Total 1972-1976	
							No.	%
2a	No. of strains	179	183	145	157	149	813	100.0
	Phage type							
	69	40.8	47.5	62.1	87.9	82.6	511	62.9
	69d	—	—	13.1	4.5	2.7	30	3.7
	71	7.3	31.7	—	—	—	71	8.7
	74	—	1.6	15.2	3.2	3.4	35	4.3
	76	17.9	8.2	0.6	0.6	1.3	51	6.3
	Other types	34.0	11.0	9.0	3.8	10.0	115	14.1
3a	No. of strains	332	403	281	253	233	1502	100.0
	Phage type							
	5	26.5	7.7	16.8	60.9	44.6	424	28.2
	18	11.1	3.0	18.2	5.1	2.2	118	7.9
	19	16.9	27.5	6.4	19.0	22.7	286	19.1
	31	5.1	21.6	12.8	0.4	6.4	156	10.4
	38	12.7	7.4	4.6	0.8	—	87	5.8
	39	0.6	9.4	—	—	0.4	41	2.7
	55	6.6	3.0	20.6	4.7	15.5	140	9.3
	Other types	20.5	20.4	20.6	9.1	8.2	250	16.6
4a	No. of strains	216	137	177	99	108	737	100.0
	Phage type							
	77	—	—	6.8	2.0	2.8	17	2.3
	81	66.2	38.7	18.0	18.2	8.3	255	34.6
	89	16.2	38.7	8.5	—	—	103	14.0
	89d	—	—	56.5	71.7	85.2	263	35.7
	90	2.3	9.5	0.6	—	—	19	2.6
	Other types	15.3	13.1	9.6	8.1	3.7	80	10.8
6	No. of strains	45	61	77	32	50	265	100.0
	Phage type							
	84	6.7	—	—	6.3	2.0	6	2.3
	85	86.6	93.4	92.2	87.4	96.0	243	91.7
	Other types	6.7	6.6	7.8	6.3	2.0	16	6.0
var. X	No. of strains	80	53	45	69	65	312	100.0
	Phage type							
	3	8.8	15.1	22.2	43.5	30.8	75	24.0
	12	27.5	34.0	48.9	13.0	9.2	77	24.7
	84	22.5	26.4	8.9	24.6	18.5	65	20.8
	Other types	41.2	24.5	20.0	18.9	41.5	95	30.5

of all shigella isolations, the epidemiological situation was determined by *S. flexneri* and *S. sonnei*. Table I shows that the number of *S. flexneri* isolations decreased gradually, whereas the number of *S. sonnei* isolations, with an intermittent decrease in 1973, showed an increasing tendency.

Within the species *S. dysenteriae*, serotype 2 occurred the most frequently; in addition, serotypes 3 and 6 were found. The species *S. boydii* was represented by serotypes 1, 8, 9, 11 and 14.

The order of frequency for the three wide-spread *S. flexneri* serotypes 3a, 2a and 4a, was the same in every year. Owing to an instruction from this Institute to all regional laboratories to extend the serotyping of *S. flexneri*, the number of untyped strains decreased gradually (Table I).

Of *S. flexneri* isolated between 1972–1976, 3881 strains were phage typed. Table II shows the phage type distribution of frequent serotypes. In serotype 2a, phage type 69 strains showed a gradual increase between 1972 and 1976, whereas the incidence of phage type 76 strains decreased. Phage types 69d, 71 and 74 occurred with higher frequency in a single year. In serotype 2a, 3 to 21, different kinds of other phage types occurred yearly.

Serotype 3a strains belonged to 7 frequent and to 11–23 infrequent phage types. Phage types 5 and 19 predominated in each year. Phage type 55 isolates were resistant to antibiotics and carried R-plasmid.

In serotype 4a, the incidence of phage type 81 strains decreased gradually, whilst phage type 89d became prevalent. Two to 11 infrequent phage types were shown.

Serotype 6 strains belonged in 91.7% to phage type 85. The rest was distributed among 8 phage types.

Variant X was represented by 3 frequent and 4 to 14 infrequent phage types.

Of *S. flexneri* isolates not shown in Table II, serotype 1b was fairly common; the 123 strains belonged in 66.4% to phage type 73, the rest to 2–7 other phage types. Less frequent serotypes were distributed as follows: 1a (21 strains, 10 different phage types), 2b (34 strains, 12 phage types), 3b and 3c (14 strains each, 6 phage types) and variant Y (30 strains, 10 phage types).

Phage and colicin typing was performed with 8271 *S. sonnei* strains. Results for phage and colicin types occurring in more than 5% of isolates are shown in Table III.

In phage type 2, colicin type 12 was the most frequent, especially in the years 1975–1976. Phage type 2, colicin non-producing strains were encountered mainly in 1972–1974.

The distribution of *S. sonnei* phage type 3 strains according to colicin types was similar to that of phage type 2 strains. Colicin non-producing strains were frequently demonstrated in phage types 6 and 7.

Table III

Distribution of frequent S. sonnei phage types according to colicin types, percentage of strains isolated in Hungary, 1972-1976

Phage type		1972	1973	1974	1975	1976	Total 1972-1976	
							No.	%
2	No. of strains	76	149	257	792	192	1466	100.0
	Colicin type							
	0	43.5	29.8	41.6	12.8	24.0	329	22.5
	2					6.8	53	3.6
	6	5.2	64.5	37.3	—	8.3	237	16.2
	12	34.3	—	9.7	75.4	55.2	757	51.7
	15	11.8	—	—	—	—	27	1.8
	Others	5.2	5.7	11.4	11.8	5.7	63	4.2
3	No. of strains	—	160	80	224	115	579	100.0
	Colicin type							
	0		34.0	58.7	13.3	38.2	175	30.3
	2		—	—	5.4	7.0	24	4.1
	3		6.9	—	—	—	16	2.7
	6		11.9	12.5	—	—	32	5.6
	11		—	5.0	—	5.2	20	3.4
	12		36.5	14.0	73.8	45.2	286	49.5
	Others		10.7	9.8	7.5	4.4	26	4.4
6	No. of strains	389	139	268	414	422	1632	100.0
	Colicin type							
	0	74.2	84.9	90.0	94.0	95.5	1434	87.9
	2	—	7.2	—	—	—	21	1.3
	15	21.8	—	5.6	—	—	102	6.2
	Others	4.0	7.9	4.4	6.0	4.5	75	4.6
7	No. of strains	165	267		233	92	757	100.0
	Colicin type							
	0	72.9	78.2		56.4	82.6	536	70.9
	2	—	5.2		—	—	24	3.1
	8	5.4	—		—	—	10	1.3
	12	10.9	7.5		30.4	7.6	115	15.2
	Others	10.8	8.6		13.2	9.8	72	9.5
21	No. of strains			71	143		214	100.0
	Colicin type							
	0			15.5	22.6		43	20.2
	6			36.7	24.0		60	28.0
	11			28.1	6.4		29	13.5
	12			11.3	16.0		32	14.9
	15			—	24.0		34	15.9
	Others			8.4	7.0		16	7.5

(contd.)

Table III (continued)

Phage type		1972	1973	1974	1975	1976	Total 1972-1976	
							No.	%
32	No. of strains	174		120			294	100.0
	Colicin type							
	0	19.5		—			34	11.5
	2	26.5		61.6			120	40.9
	4	39.1		31.7			106	36.1
	6	5.7					13	4.4
	Others	9.2		6.7			21	7.1
54	No. of strains					99	99	100.0
	Colicin type							
	0					100	99	100.0
65	No. of strains	175	122	161	433	565	1456	100.0
	Colicin type							
	0	60.0	26.2	—	15.0	16.1	299	20.4
	3a	—	—	6.8	—	—	16	1.1
	4	—	6.5	—	7.6	—	50	3.4
	6	15.5	15.6	51.1	36.3	50.6	570	39.0
	9	—	5.7	—	—	—	12	0.8
	11	6.6	27.0	23.6	18.1	5.5	192	13.2
	12	—	13.9	—	16.1	19.8	210	14.4
	Others	7.9	5.1	18.5	6.9	8.0	107	7.7

Phage type 21 exceeded the 5% incidence in 1974 and 1975, and phage type 32, in 1972 and 1974. Phage type 54 strains were isolated from one outbreak in 1976; they uniformly failed to produce colicin. The greatest variety of colicin types was encountered in phage type 65.

S. flexneri-*S. sonnei* ratio. In the five-year survey period, *S. flexneri* amounted to 40.5%, *S. sonnei* to 58.0% of all shigella isolations (Table I). An increasing incidence in Hungary of *S. sonnei* had already been observed before the survey period. In 1972 it was more frequent than *S. flexneri* only in some western and northern regions. In 1974 *S. sonnei* ranked first in the middle, southern and eastern counties, and by 1975-1976 its predominance had become characteristic of the whole country (Fig. 1).

Seasonal distribution of shigella isolations. The number of shigella isolations was, on the average, the highest in the third quarter of the year, still high in the fourth quarter and relatively low in the first and second quarters. The quarterly distribution of isolations varied considerably in each year of the survey period (Table IV).

The seasonal distribution of the two predominating shigellae was different. *S. flexneri* occurred fairly evenly throughout the year with a moderate

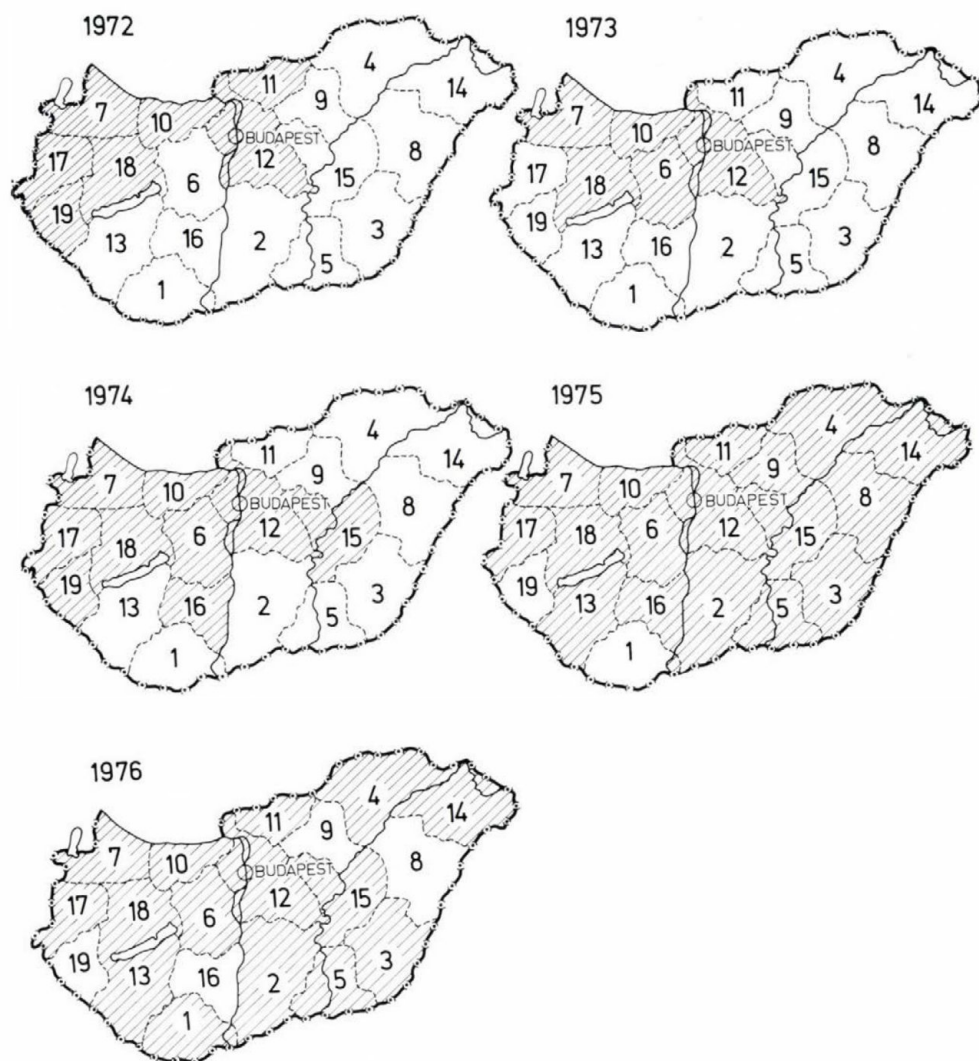


Fig. 1. Eastward trend of *S. sonnei* predominance

peak in the first quarter, whereas *S. sonnei* showed a definite predominance in the third quarter of every year. This rise in the incidence of *S. sonnei* accounted for the July–September peak of all shigella isolations (Table V).

Regional distribution of shigella isolations. The average ratio of isolation per 100 000 inhabitants ranged between 50.2 and 78.2 (Table VI). The frequency of isolations varied in the different parts of the country. The ratio was the lowest in the middle regions. In the eastern and western areas it was in each year higher than the average for the whole country. Values for the western

Table IV
Quarterly isolation of shigella in Hungary, 1972-1976

Year	January-March		April-June		July-September		October-December		Total	
	No.	%	No.	%	No.	%	No.	%	No.	%
1972	1463	20.4	1202	16.8	2602	36.3	1898	26.5	7165	100.0
1973	1375	22.3	1295	21.0	1832	29.7	1663	27.0	6165	100.0
1974	1112	20.2	976	17.7	1895	34.4	1524	27.7	5507	100.0
1975	1120	13.6	779	9.5	3750	45.5	2588	31.4	8237	100.0
1976	1318	24.8	927	17.4	1428	26.8	1652	31.0	5325	100.0
Total	6388	19.7	5179	16.0	11507	35.5	9325	28.8	32399	100.0

Table V
Quarterly isolations of S. flexneri and S. sonnei in Hungary, 1972-1976

Species and year	January-March		April-June		July-September		October-December	
	No.	%	No.	%	No.	%	No.	%
<i>S. flexneri</i>								
1972	1025	30.0	810	23.8	860	25.2	716	21.0
1973	944	29.7	846	26.7	665	20.9	721	22.7
1974	754	29.7	555	21.8	641	25.2	593	23.3
1975	664	30.9	461	21.4	596	27.7	429	20.0
1976	573	30.9	383	20.7	433	23.4	464	25.0
Total	3960	30.2	3055	23.3	3195	24.3	2923	22.3
<i>S. sonnei</i>								
1972	404	11.1	363	10.0	1717	47.1	1161	31.8
1973	404	14.1	421	14.7	1141	39.8	903	31.4
1974	329	11.4	387	13.5	1237	43.1	918	32.0
1975	440	7.3	304	5.1	3100	51.7	2150	35.9
1976	724	21.3	527	15.5	977	28.7	1171	34.5
Total	2301	12.2	2002	10.7	8172	43.5	6303	33.6

regions were in three years somewhat higher than the country average. In Budapest, the ratio of isolations for 100 000 inhabitants was constantly lower, in 1974 and 1976, significantly lower, than the country average.

Table VI

Incidence of shigella isolations per 100 000 inhabitants in different geographic regions of Hungary, 1972-1976

Geographic region	1972	1973	1974	1975	1976
West ¹	77.6	64.2	62.4	72.1	45.9
Middle ²	41.5	33.8	21.6	46.4	29.2
East ³	138.9	66.7	63.0	101.6	86.0
North ⁴	71.9	74.7	83.7	97.2	66.7
Budapest	65.4	50.9	27.6	70.3	19.2
Total	69.1	59.2	52.7	78.2	50.2

¹ Counties Baranya, Fejér, Győr-Sopron, Komárom, Somogy, Tolna, Vas, Veszprém, Zala

² Counties Bács-Kiskun, Pest

³ Counties Békés, Csongrád, Hajdú-Bihar, Szabolcs-Szatmár, Szolnok

⁴ Counties Heves, Nógrád, Borsod-Abaúj-Zemplén

Shigella isolations from epidemic outbreaks and from sporadic cases. In the five-year survey period more shigellae were isolated from sporadic cases than from outbreaks (54.7% vs. 45.3%). This distribution was characteristic of each year except 1976 (Table VII).

As shown in Table VIII, *S. flexneri* occurred more frequently in each year in sporadic cases than in outbreaks. The five-year average of *S. sonnei* isolations was, however, almost the same for sporadic cases and outbreaks. This similar average of epidemic and sporadic isolations of *S. sonnei* was associated with an extensive milk-borne outbreak in 1976, shifting the ratio

Table VII

Shigella isolations from epidemics and sporadic cases, 1972-1976

Year	Epidemic cases		Sporadic cases		Total	
	No.	%	No.	%	No.	%
1972	3235	45.2	3930	54.8	7165	100.0
1973	2630	42.7	3535	57.3	6165	100.0
1974	2481	45.1	3026	54.9	5507	100.0
1975	3467	42.1	4770	57.9	8237	100.0
1976	2879	54.1	2446	45.9	5325	100.0
Total	14692	45.3	17707	54.7	32399	100.0

Table VIII

Isolations of S. flexneri and S. sonnei from epidemics and sporadic cases, 1972-1976

Species and year	Epidemic cases		Sporadic cases		Total	
	No.	%	No.	%	No.	%
<i>S. flexneri</i>						
1972	1514	44.4	1897	55.6	3411	100.0
1973	1329	41.8	1847	58.2	3176	100.0
1974	1062	41.8	1481	58.2	2543	100.0
1975	859	40.0	1291	60.0	2150	100.0
1976	763	41.2	1090	58.8	1853	100.0
Total	5527	42.1	7606	57.9	13133	100.0
<i>S. sonnei</i>						
1972	1696	46.5	1949	53.5	3645	100.0
1973	1269	44.2	1600	55.8	2869	100.0
1974	1404	48.9	1467	51.1	2871	100.0
1975	2569	42.9	3425	57.1	5994	100.0
1976	2100	61.8	1299	38.2	3399	100.0
Total	9038	48.1	9740	51.9	18778	100.0

Table IX

Shigella isolations from patients and asymptomatic excretors, 1973-1976

Year	Total no.	Shigella isolations				Patients/ excretors
		Patients		Excreters		
		No.	%	No.	%	
1973	6165	3924	63.6	2241	36.4	1.8: 1
1974	5507	3465	62.9	2042	37.1	1.7: 1
1975	8237	5532	67.2	2705	32.8	2.0: 1
1976	5325	3246	61.0	2079	39.0	1.6: 1
Total	25234	16167	64.1	9067	35.9	1.8: 1

of epidemic *S. sonnei* isolations to the unusually high value of 61.8%. In other years, the ratio of *S. sonnei* isolations was higher for sporadic than for epidemic cases.

Shigella isolations from patients and asymptomatic excretors. Nation-wide data have been collected since 1973 (Table IX). Out of the 25 234 shigella

isolations in 1973–1976, 64.1% belonged to patients and 35.9% to excreters. A closely similar distribution was characteristic of each year of the survey period.

Table X shows that there was no great difference in the number of *S. flexneri* isolations from patients and from excreters. In contrast, about $\frac{2}{3}$ of *S. sonnei* isolations originated from patients.

Table X

S. flexneri and *S. sonnei* isolations from patients and asymptomatic excreters, 1973–1976

Species and year	Total no.	Shigella isolation				Patients/ excreters
		Patients		Excreters		
		No.	%	No.	%	
<i>S. flexneri</i>						
1973	3176	1811	57.0	1365	43.0	1.3 : 1
1974	2543	1338	52.6	1205	47.4	1.1 : 1
1975	2150	1174	54.6	976	45.4	1.2 : 1
1976	1853	987	53.3	866	46.7	1.1 : 1
Total	9722	5310	54.6	4412	45.4	1.2 : 1
<i>S. sonnei</i>						
1973	2869	2045	71.3	824	28.7	2.5 : 1
1974	2871	2079	72.4	792	27.6	2.6 : 1
1975	5994	4290	71.6	1704	28.4	2.5 : 1
1976	3399	2216	65.2	1183	34.8	1.9 : 1
Total	15133	10630	70.2	4503	29.8	2.4 : 1

Outbreaks of shigellosis. In the period between 1972 and 1976, 3279 epidemics involving 14 692 cases were reported (Table XI).

The distribution of epidemics according to *S. flexneri* and *S. sonnei* is shown in Table XII. The number of epidemics and cases associated with *S. flexneri* decreased gradually during the five-year period. The case/epidemic ratio, too, showed a decreasing tendency. The incidence of *S. sonnei* epidemics and cases varied with the years of the survey period. The average case/epidemic ratio was somewhat higher for *S. sonnei* than for *S. flexneri*.

In 574 out of the 3279 epidemics considerably more cases were reported than the average (Table XIII). A total of 176 non-community outbreaks was associated mostly with *S. sonnei*. The rest was reported in healthy children communities (276 outbreaks, 4934 cases), in residential homes for the aged

Table XI*Number of dysentery epidemics and cases confirmed by isolation, 1972-1976*

Year	Epidemics		Cases per epidemic
	No.	Cases	
1972	744	3235	4.4
1973	643	2630	4.1
1974	618	2481	4.0
1975	808	3467	4.3
1976	466	2879	6.2
Total	3279	14692	4.5

Table XII*Number of S. flexneri and S. sonnei epidemics and cases confirmed by isolation, 1972-1976*

Species and year	Epidemics		Cases per epidemic
	No.	Cases	
<i>S. flexneri</i>			
1972	356	1514	4.3
1973	328	1329	4.1
1974	283	1062	3.8
1975	237	859	3.6
1976	194	763	3.9
Total	1398	5527	4.0
<i>S. sonnei</i>			
1972	376	1696	4.5
1973	306	1269	4.1
1974	328	1404	4.3
1975	567	2569	4.5
1976	269	2100	7.8
Total	1846	9038	4.9

and mental asylums (106 outbreaks, 1239 cases), and in various hospitals (16 outbreaks, 324 cases). The majority of the epidemics was caused by *S. sonnei*, but in mental institutions and aged people's homes *S. flexneri* predominated.

Table XIII
Important dysentery epidemics in Hungary, 1972-1976

Epidemics	No. of epidemics	No. of cases	Causative agent		
			<i>S. flexneri</i>	<i>S. sonnei</i>	Other <i>Shigella</i> species
1. <i>Non-community epidemics</i>	176	4674	58	117	1
2. <i>Community epidemics</i>					
Day nurseries	29	309	8	21	—
Kindergartens	114	2150	15	99	—
Infants homes	42	469	11	31	—
Children's homes	18	269	9	9	—
Primary schools	32	1115	8	23	1
Colleges	41	622	14	27	—
Homes for mentally retarded children	46	467	28	18	—
Homes for aged persons	39	585	33	5	1
Mental wards	21	187	19	2	—
Other wards	16	324	2	14	—
Total	574	11 171	205	366	3

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KLEBSIELLA AND ENTEROBACTER STRAINS DERIVED FROM HOSPITAL INFECTIONS

I. CORRELATION BETWEEN SPECIES, PHAGE TYPE AND ANTIBIOTIC SENSITIVITY

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Four hundred and seventy *Klebsiella* and 103 *Enterobacter* strains derived from urogenital infections, upper respiratory tract and wound infections were examined. (i) *K. aerogenes* was the most common among *Klebsiella* species, *K. ozaenae* and *K. atlantae* occurred frequently, *K. pneumoniae*, *K. rhinoscleromatis*, *K. edwardsii* and *K. oxytoca* were found rarely. The strains isolated from urine showed the most heterogeneous species distribution. *Enterobacter* species were in the order of frequency *E. cloacae*, *E. liquefaciens*, *E. aerogenes*. (ii) *K. aerogenes* strains belonged to 10, *K. atlantae* strains to 6 phage types; phage type II.A1 was the most frequent in both species. *K. ozaenae*, *K. pneumoniae* and *K. rhinoscleromatis* were divided into four phage types. Typability varied between 23.7% and 48.0% with the different *Klebsiella* species, except *K. edwardsii* and *K. oxytoca* strains. None of the strains of these species were typable by phages. Among the *Enterobacter* species examined, only one *E. liquefaciens* strain was typable by phages. Strains of genus *Enterobacter* were lysed by the diagnostic phage C14 in 64%, no lysis was observed in the strains of genus *Klebsiella*. (iii) Multiple resistance was demonstrated in 36.6% of *Klebsiella* strains and in 16.5% of *Enterobacter* strains. The majority of the multiresistant strains was isolated from urine in both genera. Multiresistant strains belonged to the species *K. aerogenes*, *E. cloacae* and *E. liquefaciens*. Strains resistant to ampicillin were divided into 6 groups on the basis of cephaloridine and cephalothin resistance. The rate of multiresistant strains was higher among the *Klebsiella* and *Enterobacter* strains not typable by phages than in the typable ones.

Though *Klebsiella* and *Enterobacter* are similar in fenotype, they differ in genetic fine structure [1]. Strains of the genus *Klebsiella* were more frequent in severe hospital outbreaks than those belonging to genus *Enterobacter*. Multiple antibiotic resistance was more often reported in the genus *Klebsiella* than in *Enterobacter*. For practical purposes, strains of the tribus *Klebsiellae* have not been routinely classified according to genus and species and thus important epidemiological connections have remained hidden. To elaborate directives for prevention and control of the spread of infections, examinations according to different aspects of the pathogenic organism isolated from infections were needed. The present study deals with the classification according to species completed with phage typing and antibiotic sensitivity tests.

Materials and methods

Bacterial strains. A total of 573 strains belonging to *Klebsiella* were examined for species, phage type and antibiotic sensitivity. Out of the examined strains 470 belonged to *Klebsiella* and 103 to *Enterobacter* on the basis of motility and ornithine decarboxylase test.

The strains were derived from in-patients of hospitals and hospital wards (surgical, urological, infant and premature) from different conditions (urogenital infections, upper respiratory tract diseases, enteritis, wound infections), from different materials (urine, faeces, secretions from nose, throat and wounds). These strains were isolated by the County Public Health

Stations and the Public Health Station of the City of Budapest from January 1, 1976 to June 30, 1978.

Strains used for propagation of phages. For propagation, the strains isolated by MILCH and DEÁK [2] and SLOPEK *et al.* [3] were used.

Phages. The phages described by SLOPEK *et al.* [3] for phage typing of *Klebsiella* strains were used (basic phages I–XV and additional phages 4, 7 and 35). The diagnostic *Enterobacter* phage was used according to MILCH and DEÁK [2].

Media. For phage propagation and typing beef-extract agar, for antibiotic sensitivity tests a modified Mueller–Hinton agar was used [4, 5].

Classification according to species. Classification of *Klebsiella* and *Enterobacter* species was carried out on the basis of biochemical tests: urea, indole, methyl red (37 °C), Voges–Proskauer (22 °C), ammonium citrate (37 °C), lysine decarboxylase and lactose [6].

Determination of phage type. Phage typing was performed according to SLOPEK *et al.* [3]. Phages were used in routine test dilutions (RTD) i.e. the highest dilution which produced confluent lysis on the homologous strain. Several colonies were picked up from the agar plate and inoculated into broth. After 2 h incubation at 37 °C the culture was seeded onto agar medium the excess being drawn off and the type-phages were spotted on the plates by a phage typing apparatus. After the drops had dried the plates were incubated at 37 °C overnight. After reading the lytic results, phage types were determined according to the typing-scheme.

Antibiotic sensitivity was determined to ampicillin (Ap), streptomycin (Sm), chloramphenicol (Cm), tetracycline (Tc), kanamycin (Km), neomycin (Nm), polymyxin-B (Pm), colistin (Co), gentamicin (Gm) and nalidixic acid (Nal) by the use of "Resistest" disks (Institute for Serobacteriological Production and Research Human, Budapest). A total 36 *Klebsiella* and 29 *Enterobacter* strains were tested by the disk method for cephaloridine and cephalothin sensitivity.

Results

Classification and incidence of species. The examined 470 *Klebsiella* strains belonged to 7 species on the basis of biochemical tests (Table I). The most common was *K. aerogenes*, *K. ozaenae* and *K. atlantae* occurred frequently, the other species were rather rare. Out of examined 102 *Enterobacter* strains

Table I
Classification of *Klebsiella* and *Enterobacter* species on the basis of biochemical reactions

Biochemical reactions	<i>K. aerogenes</i>	<i>K. atlantae</i>	<i>K. edwardsii</i>	<i>K. oxytoca</i>	<i>K. ozaenae</i>	<i>K. pneumoniae</i>	<i>K. rhinoscleromatis</i>	<i>E. aerogenes</i>	<i>E. cloacae</i>	<i>E. liquefaciens</i>
Urea	+	+	+	+	d	+	—	—	d	d
Methyl red 37 °C	—	+	d	—	+	+	+	—	—	d
Voges–Proskauer 22 °C	+	d	+	+	—	—	—	+	+	+
Ammonium citrate 37 °C	+	+	—	+	d	+	—	+	+	+
Lysine decarboxylase	+	+	+	+	d	+	—	+	—	—
Indole	—	—	—	+	—	—	—	—	—	—
Lactose	+	+	+	+	+	+	—	+	+	+
Strains examined										
No.	372	25	9	3	38	13	10	4	79	20
%	79.2	5.3	1.9	0.6	8.1	2.8	2.1	3.9	76.9	19.4

d = different

Table II

Distribution in percentage of Klebsiella and Enterobacter species according to the source of isolation

Source of isolation	<i>K. aerogenes</i>	<i>K. atlantae</i>	<i>K. edwardsii</i>	<i>K. oxytoca</i>	<i>K. ozaenae</i>	<i>K. pneumoniae</i>	<i>K. rhinoscleromatis</i>	Klebsiella total		<i>E. aerogenes</i>	<i>E. cloacae</i>	<i>E. liquefaciens</i>	Enterobacter total	
								No.	%				No.	%
Nose	6.0	—	—	—	0.4	—	—	30	6.4	—	13.6	—	14	13.6
Throat	10.0	0.2	—	0.4	2.6	0.2	—	63	13.4	—	22.3	—	23	22.3
Faeces	16.0	—	—	—	0.2	—	—	76	16.2	—	—	—	—	—
Urine	27.4	3.6	1.5	—	3.8	2.6	1.7	191	40.6	—	17.5	18.4	37	35.9
Fomites, baby food etc.	19.8	1.5	0.4	0.2	1.1	—	0.4	110	23.4	3.9	23.3	1.0	29	28.2
Total	79.2	5.3	1.9	0.6	8.1	2.8	2.1	470	100.0	3.9	76.7	19.4	103	100.0

Table III

Distribution in percentage of Klebsiella and Enterobacter species according to origin

Origin of strains	<i>K. aerogenes</i>	<i>K. atlantae</i>	<i>K. edwardsii</i>	<i>K. oxytoca</i>	<i>K. ozaenae</i>	<i>K. pneumoniae</i>	<i>K. rhinoscleromatis</i>	Klebsiella total		<i>E. aerogenes</i>	<i>E. cloacae</i>	<i>E. liquefaciens</i>	Enterobacter total	
								No.	%				No.	%
H. 1.	66.3	12.7	4.2	—	8.4	4.2	4.2	95	20.2	—	33.3	66.7	21	20.4
H. 2.	94.4	—	5.6	—	—	—	—	18	3.8	—	43.0	57.0	7	6.8
H. 3.	80.2	6.3	0.8	1.6	7.9	2.4	0.8	126	26.8	3.5	96.5	—	57	55.4
H. 4	93.8	1.2	—	—	3.8	—	1.2	79	16.8	22.2	77.8	—	9	8.7
H. others	77.0	2.6	2.0	0.8	11.1	3.9	2.6	152	32.4	—	77.8	22.2	9	8.7
Total	79.2	5.3	1.9	0.6	8.1	2.8	2.1	470	100.0	3.9	76.7	19.4	103	100.0

H. 1 = Urological ward, Budapest

H. 2 = Surgical ward, Budapest

H. 3 = Infant and premature infant ward, County Hospital No. 1

H. 4 = Premature infant ward, County Hospital No. 2

H. others = different hospital wards

Table IV
Phage type distribution of Klebsiella

Species	Phage type											
	I. A1	II. A1	III. A1	IV. A1	V. A1	VI. A1	VII. A1	VIII. A1	IX. A1	X. A1	XI. A1	XII. A1
<i>K. aerogenes</i>	7	83	3	21	3	1	10	11	—	—	—	1
<i>K. atlantae</i>	2	4	1	—	—	—	1	1	—	—	—	—
<i>K. edwardsii</i>	—	—	—	—	—	—	—	—	—	—	—	—
<i>K. oxytoca</i>	—	—	—	—	—	—	—	—	—	—	—	—
<i>K. ozaenae</i>	—	3	—	—	1	1	—	4	—	—	—	—
<i>K. pneumoniae</i>	1	1	—	—	—	—	1	—	—	1	—	—
<i>K. rhinoscleromatis</i>	1	—	1	—	—	—	1	1	—	—	—	—
Total <i>Klebsiella</i> no. %	11 2.3	91 19.4	5 1.1	21 4.5	4 0.9	2 0.4	13 2.8	17 3.6	—	1 0.2	—	1 0.2
Total <i>Enterobacter</i> no. %	1 0.9	—	—	—	—	—	—	—	—	—	—	—

E. cloacae was the most frequent, *E. liquefaciens* was frequent and *E. aerogenes* was rare.

Table II shows the species distribution of *Klebsiella* and *Enterobacter* strains in per cent according to the source of isolation. The most common was *K. aerogenes* in all tested materials. The occurrence of *K. ozaenae*, *K. atlantae*, *K. pneumoniae*, *K. edwardsii* and *K. rhinoscleromatis* among the strains isolated from urine was rare. Strains isolated from nasal samples and faeces could be classified as *K. aerogenes* and *K. ozaenae*. Besides these two frequent species, *K. atlantae*, *K. oxytoca* and *K. pneumoniae* were isolated from throats. All species except *K. pneumoniae* were found among the strains isolated from fomites and from baby food. All of the *Enterobacter* strains isolated from nose and throat samples were classified as *E. cloacae*. The strains derived from urine belonged to *E. cloacae* and *E. liquefaciens*.

Table III shows the species distribution of *Klebsiella* and *Enterobacter* strains according to the site of isolation. Strains derived from surgical wards belonged to *K. aerogenes* in 94.4%. All *Klebsiella* species except *K. oxytoca* were found in urological wards. Three *K. oxytoca* strains were identified (8.4%), two of them were isolated from a patient in the premature infant ward in a county hospital. The strains derived from urological and surgical wards were classified as *E. cloacae* and *E. liquefaciens*. *E. aerogenes* was also demonstrated among the strains isolated in county hospitals.

and *Enterobacter* species

XIII. A1	XIV. A1	XV. A1	Additional phages				Typable		Not typable		Total no.
			K 4	K 7	K 35	K 4, 7	No.	%	No.	%	
—	—	1	5	3	2	3	154	41.4	218	58.6	372
—	—	2	—	1	—	—	12	48.0	13	52.0	25
—	—	—	—	—	—	—	—	—	9	100.0	9
—	—	—	—	—	—	—	—	—	3	100.0	3
—	—	—	—	—	—	—	9	23.7	29	76.3	38
—	—	—	—	—	—	—	4	30.8	9	69.2	13
—	—	—	—	—	—	—	4	40.0	6	60.0	10
—	—	3	5	4	2	3	183	38.9	287	61.1	470
—	—	0.6	1.1	0.9	0.4	0.6					
—	—	—	—	—	7	—					
—	—	—	—	—	6.9	—	8	7.8	95	92.2	103

Phage type distribution. Table IV presents the phage type distribution of *Klebsiella* and *Enterobacter* strains. *Klebsiella* strains belonged to phage type II.A1 in 19.4%, the other phage types were rare. Typability of the strains was 38.9%. *K. aerogenes* strains were divided into 10 phage types, the most frequent type was II.A1; by the use of additional phages four other groups could be distinguished.

K. atlantae strains belonged to 6 phage types; phage type II.A1 was the most common among the strains of this species. *K. ozaenae* and *K. rhinoscleromatis* strains were divided into four phage types. Most of the *K. ozaenae* strains were of phage type VIII.A1. The different *Klebsiella* species were typable in 23.7–48.0%, while no *K. edwardsii* and *K. oxytoca* strains were typable. Out of the different *Enterobacter* strains, one *E. liquefaciens* strain was typable, it belonged to phage type I.A1. Seven strains were sensitive to *Klebsiella* phage K35.

Table V shows the phage type distribution of strains according to the source of isolation. Phage type II.A1 was predominant among the strains derived from nasal, throat and faecal samples, whereas the strains isolated from urine showed more heterogeneous phage types. Strains derived from nasal, throat and faecal samples belonged to 3–4 phage types, those isolated from urine, to 11 phage types. Analysing the phage type distribution according to hospitals (Table VI), the predominance of phage type II.A1 was the most

Table V
Phage type distribution of Klebsiella strains according to the source of isolation

Material	Phage type															Additional phages				Total	
	I. A1	II. A1	III. A1	IV. A1	V. A1	VI. A1	VII. A1	VIII. A1	IX. A1	X. A1	XI. A1	XII. A1	XIII. A1	XIV. A1	XV. A1	K 4	K 7	K 35	K 4,7	No.	%
Nose	—	9	—	1	—	—	—	3	—	—	—	—	—	—	—	—	—	—	—	13	7.1
Throat	—	17	2	—	—	—	1	3	—	—	—	—	—	—	—	—	—	—	—	23	12.6
Faeces	—	17	—	4	—	—	2	3	—	—	—	—	—	—	—	—	3	—	1	30	16.4
Urine	8	19	3	14	3	2	7	7	—	1	—	1	—	—	1	4	—	2	—	72	39.3
Others	3	29	—	2	1	—	3	1	—	—	—	—	—	—	2	1	1	—	2	45	24.6
Total	11	91	5	21	4	2	13	17	—	1	—	1	—	—	3	5	4	2	3	183	100.0

Table VI
Phage type distribution of Klebsiella strains according to origin

Origin of strains	Phage types															Additional phages				Total	
	I. A1	II. A1	III. A1	IV. A1	V. A1	VI. A1	VII. A1	VIII. A1	IX. A1	X. A1	XI. A1	XII. A1	XIII. A1	XIV. A1	XV. A1	K 4	K 7	K 35	K 4,7	No.	%
H. 1	7	11	1	9	—	—	6	3	—	—	—	—	—	—	—	—	—	—	—	37	20.2
H. 2	1	2	1	3	2	—	1	—	—	—	—	—	—	—	1	1	—	—	—	12	6.6
H. 3	1	58	2	5	1	—	3	—	—	—	—	1	—	—	2	1	—	—	2	76	41.5
H. 4	—	10	—	4	—	—	1	6	—	1	—	—	—	—	—	2	2	—	—	26	14.2
H. others	2	10	1	—	1	2	2	8	—	—	—	—	—	—	—	1	2	2	1	32	17.5
Total	11	91	5	21	4	2	13	17	—	1	—	1	—	—	3	5	4	2	3	183	100.0

H. 1 = Urological ward, Budapest

H. 2 = Surgical ward, Budapest

H. 3 = Infant and premature infant ward, County Hospital No. 1

H. 4 = Premature infant ward, County Hospital No. 2

H. others = different hospital wards

Table VII

Lytic reactions of Klebsiella and Enterobacter strains by diagnostic phage, C14

Species, genus	No. of strains examined	Strains lysed by phage C14		Strains not lysed by phage C14	
		No.	%	No.	%
<i>E. aerogenes</i>	4	—	—	4	100.0
<i>E. cloacae</i>	79	61	77.2	18	22.8
<i>E. liquefaciens</i>	20	5	25.0	15	75.0
<i>Enterobacter</i> total	103	66	64.0	37	36.0
<i>Klebsiella</i> total	470	—	—	470	100.0

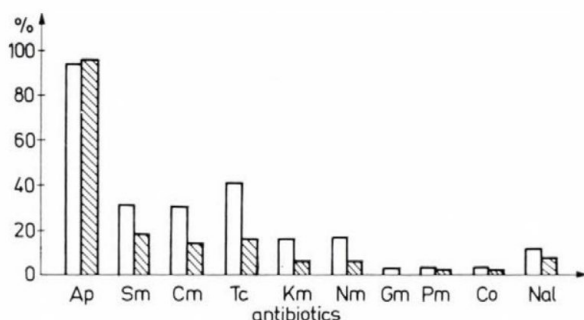


Fig. 1. Occurrence of antibiotic resistance determinants among 470 *Klebsiella* and 103 *Enterobacter* strains. Open columns: resistance determinants of *Klebsiella* strains, per cent; shaded columns: resistance determinants of *Enterobacter* strains, per cent

significant in H3 (county hospital), and this phage type was the most frequent in the other hospitals too. In a hospital 6 to 11 phage types could be demonstrated.

Enterobacter strains were not typable by phages, only one strain, isolated from the throat of a patient in a county hospital (H3), could be ranged in a phage type.

Sensitivity to diagnostic phage of Klebsiella and Enterobacter strains. Table VII presents the lytic reactions of diagnostic phage C14 used to separate the two genera. *Enterobacter* strains were lysed by phage C14 in 64.0%; the rate of *E. cloacae* strains lysed by phage C14 was higher (77.2%) than that of the *E. liquefaciens* strains (25.0%). Analysing the phage sensitivity according to the material and source of infection it was found that the strains isolated from throat in a county hospital (H3) were lysed in the highest per cent. Of the examined 470 *Klebsiella* strains not a single one was lysed by phage C14.

Tests of antibiotic sensitivity. Figure 1 shows the resistance to antibiotics of 470 *Klebsiella* and 103 *Enterobacter* strains; they were resistant to ampicillin

Table VIII
Distribution of Klebsiella species according to antibiotic resistance

Antibiotics	<i>K. aerogenes</i>	<i>K. atlantae</i>	<i>K. edwardsii</i>	<i>K. oxytoca</i>	<i>K. ozaenae</i>	<i>K. pneumoniae</i>	<i>K. rhinoscleromatis</i>	Total
Ap	350	25	9	3	36	10	9	442
Sm	114	9	5	—	7	7	7	149
Cm	108	10	6	—	9	7	6	146
Tc	148	13	6	—	12	6	6	191
Km	51	10	2	—	7	2	2	74
Nm	53	9	2	—	7	2	2	75
Gm	2	2	—	—	—	1	—	5
Pm	2	1	—	—	—	1	—	4
Co	14	—	—	—	—	1	—	15
Nal	39	9	7	—	5	3	3	66
No. of strains examined	372	25	9	3	38	13	10	470

in 94% and 95%, respectively. To other antibiotics, *Klebsiella* strains were resistant in a higher degree than *Enterobacter* strains (except polymyxin-B).

Table VIII presents the resistance to antibiotics of the different *Klebsiella* species. The majority of the strains of all species — with the exception of *K. pneumoniae* — were resistant to ampicillin. Resistance to streptomycin and chloramphenicol was frequent among the strains of *K. edwardsii*, *K.*

Table IX
Distribution of Enterobacter species according to antibiotic resistance

Antibiotics	<i>K. aerogenes</i>	<i>K. cloacae</i>	<i>K. liquefaciens</i>	Total
Ap	4	77	17	98
Sm	—	4	14	18
Cm	—	4	11	15
Tc	—	4	12	16
Km	—	2	4	6
Nm	—	2	4	6
Gm	—	—	—	—
Pm	—	—	1	1
Co	—	2	—	2
Nal	—	1	7	8
No. of strains examined	4	79	20	103

Table X

Grouping of multiresistant *Klebsiella* and *Enterobacter* strains according to the source of isolation

Source of isolation	No. of <i>Klebsiella</i> strains examined	Multiresistant strains %	No. of <i>Enterobacter</i> strains examined	Multiresistant strains %
Nose	30	26.7	14	—
Throat	63	19.0	23	—
Faeces	76	14.5	—	—
Urine	191	66.0	37	46.0
Others	110	13.6	29	—
Total	470	36.6	103	16.5

pneumoniae and *K. rhinoscleromatis*, and to tetracycline, of *K. edwardsii* and *K. rhinoscleromatis*. Kanamycin and gentamicin resistance was the most frequent in *K. atlantae*. There was a significant difference in kanamycin resistance between *K. aerogenes* and *K. atlantae* strains ($P < 0.001$). Resistance to polymyxin-B and colistin was frequent among the *K. pneumoniae* strains and to nalidixic acid among the *K. edwardsii* and *K. atlantae* strains. Thus, strains of the genus *Klebsiella* were most often resistant to ampicillin, chloramphenicol, tetracycline and streptomycin.

Table IX demonstrates the resistance of *Enterobacter* species. *E. aerogenes* strains were resistant only to ampicillin, *E. cloacae* strains also to other antibiotics, but all of the strains were sensitive to gentamicin and polymyxin-B. *E. liquefaciens* strains were mostly resistant beside ampicillin to streptomycin, chloramphenicol, tetracycline and nalidixic acid. Strains of the genus *Enterobacter* were mainly resistant to ampicillin, in a smaller degree to streptomycin, chloramphenicol, tetracycline and nalidixic acid and seldom to kanamycin,

Table XI

Multiresistant *Klebsiella* and *Enterobacter* strains in percentage according to the origin of strains

Origin of strains	No. of <i>Klebsiella</i> strains examined	Multiresistant strains %	No. of <i>Enterobacter</i> strains examined	Multiresistant strains %
Urological ward	95	83.2	21	61.9
Surgical ward	18	61.0	7	42.9
Infant and premature infant ward in County Hospital No. 1	125	12.0	57	—
Premature infant ward in County Hospital No. 2	79	20.0	9	—
Different hospital wards	153	33.3	9	11.0
Total	470	36.6	103	16.5

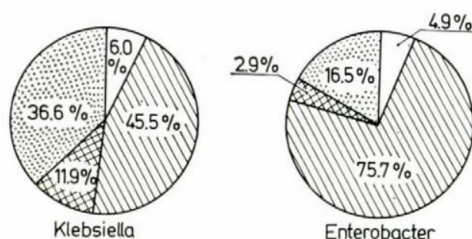


Fig. 2. Resistance to antibiotics of *Klebsiella* and *Enterobacter* strains, per cent. Open area: sensitive to antibiotics = 6.0–4.9; shaded area: monoresistant = 45.5–75.7; hatched area: double resistant = 11.9–2.9; stippled area: multiple resistant = 36.6–16.5

neomycin, colistin and polymyxin-B. *Enterobacter* strains resistant to gentamicin were not found. (Tables VII–IX contain the data in number and per cent, because of the small number of strains in one group.) *Klebsiella* strains were sensitive to the ten antibiotics tested in 6.0%, they were monoresistant in 45.5%, double resistant in 11.9% and multiple resistant in 36.6%. *Enterobacter* strains were monoresistant in 75.7%, double resistant in 2.9% and multiple resistant in 16.5% (Fig. 2).

As shown in Table X, most multiresistant strains were found in urine both for *Klebsiella* and for *Enterobacter*, though the rate was lower for the latter (66.0 vs. 46.0).

Table XII

Multiresistant strains among *Klebsiella* and *Enterobacter*

	No. of strains examined	Multiresistant strains	
		No.	%
<i>K. aerogenes</i>	372	129	34.7
<i>K. atlantae</i>	25	13	52.0
<i>K. edwardsii</i>	9	6	67.0
<i>K. oxytoca</i>	3	—	—
<i>K. ozaenae</i>	38	10	26.3
<i>K. pneumoniae</i>	13	7	53.8
<i>K. rhinoscleromatis</i>	10	7	70.0
<i>Klebsiella</i> total	470	172	36.6
<i>E. aerogenes</i>	4	—	—
<i>E. cloacae</i>	79	4	5.1
<i>E. liquefaciens</i>	20	13	65.0
<i>Enterobacter</i> total	103	17	16.5

Table XIII

Grouping of 36 monoresistant (ampicillin) *Klebsiella* and 29 monoresistant (ampicillin) *Enterobacter* strains on the basis of resistance to β -lactam antibiotics

Antibiotics	Resistance groups					
	1	2	3	4	5	6
	Zone of inhibition in mm					
Ampicillin	0	6-10	0	6-10	6-10	0
Cephaloridine	0	0	0	0	0	0
Cephalothin	0	0	10-17	25	10-17	25
No. of <i>Klebsiella</i> strains examined	7	7	4	9	1	8
No. of <i>Enterobacter</i> strains examined	5	13	3	3	4	1

As to the origin of the strains (Table XI) the majority of multiresistant *Klebsiella* and *Enterobacter* strains were derived from an urological ward, the strains isolated in a surgical ward were next in order. The strains obtained from infant and premature infant wards were resistant to one or two antibiotics.

The incidence of multiresistance among the species was also studied (Table XII). *K. rhinoscleromatis* and *K. edwardsii* strains proved to be multiresistant in the highest degree. Though in some species only small numbers were tested, the difference in multiresistance between *K. rhinoscleromatis*, *K. edwardsii* and other *Klebsiella* species was significant ($P \leq 0.05$). Among the *Enterobacter* species multiresistance occurred most often in *E. liquefaciens*.

Distribution of ampicillin monoresistant strains on the basis of cephaloridine and cephalothin resistance. On the basis of beta-lactamase-bound resistance, 36 ampicillin resistant *Klebsiella* and 29 *Enterobacter* strains were studied for cephaloridine and cephalothin resistance by examining the zones of inhibition. The strains resistant solely to ampicillin could be divided into 6 groups on the basis of their cephaloridine and cephalothin sensitivity (Table XIII).

Table XIV

Connection between antibiotic resistance and typability by phages among *Klebsiella* strains

	Antibiotic sensitive		Monoresistant		Double resistant		Multiple resistant		Total	
	No.	%	No.	%	No.	%	No.	%	No.	%
Typable	3	10.7	118	55.1	12	21.4	50	29.1	183	38.9
Not typable	25	89.3	96	44.9	44	78.6	122	70.9	287	61.1
Total	28	100.0	214	100.0	56	100.0	172	100.0	470	100.0

Correlation between antibiotic sensitivity and phage type. Examining *Klebsiella* strains of different phage types, multiresistant strains were often demonstrated in phage types II.A1, III.A1, V.A1 and VI.A1.

Antibiotic sensitive strains were typable in 10.7% and multiresistant ones in 29.1%. Table XIV shows the incidence of multiresistance among the typable and not typable *Klebsiella* strains.

Enterobacter strains were lysed by diagnostic phage C14 in 64.0%. Mono-resistant strains were lysed in 74.4%, double-resistant strains in 66.7% and multiresistant ones in 35.5% (Table XV).

Table XV

Connection between antibiotic resistance and lysis by diagnostic phage C14 of Enterobacter strains

	Antibiotic sensitive		Monoresistant		Double resistant		Multiple resistant		Total	
	No.	%	No.	%	No.	%	No.	%	No.	%
No. of strains lysed by C14	—	—	58	74.4	2	66.7	6	35.3	66	64.0
No. of strains not lysed by C14	5	100.0	20	25.6	1	33.3	11	64.7	37	36.6
Total	5	100.0	78	100.0	3	100.0	17	100.0	103	100.0

Discussion

K. aerogenes strains amounted to 80% in the genus *Klebsiella*. From nose and faeces only *K. aerogenes* and *K. ozaenae* could be isolated, from throat washings and urine the number of isolated species was 5 and 6, respectively. The rare species were responsible mainly for pathogenic urinary processes. *K. oxytoca* strains isolated from throat were obtained from in-patients of two different hospitals, and the presence of the strains was demonstrated in the surroundings, too. There are few data on the pathogenic role and frequency of the species.

Strains of *E. cloacae* made up 76.7% of the genus *Enterobacter*. The presence of other species could be demonstrated only from urine and faecal specimens. Infections, mainly in urological and surgical wards, were caused by *E. liquefaciens*, a species not or rarely isolated from infant wards.

COOKE *et al.* [7] isolated *K. aerogenes* in 98% from different materials of hospital patients. They found the presence in urine of *K. pneumoniae*, *K. edwardsii* and *K. ozaenae* strains remarkable. In our material the occurrence of *K. aerogenes* was only 79.2% and the frequency of infections caused by the other species was higher. *K. atlantae* and *K. rhinoscleromatis* strains were also demonstrated from urine besides *K. pneumoniae*, *K. edwardsii* and *K. ozaenae*.

SLOPEK [8] reported that *K. aerogenes* strains were typable by phages in 61.9%, *K. pneumoniae* strains in 72.5%. *K. ozaenae* strains were not typable by phages. The data published on the typability of *K. rhinoscleromatis* strains were not given in per cent, the strains belonged to 8 different phage types.

MILCH and DEÁK [2] determined the phage type of 802 *Klebsiella* strains and the strains were typable in 68%. Strains derived from hospital outbreaks were typable in 73%, strains of sporadic origin in 40%. PÁLL *et al.* [9] found that *Klebsiella* strains were typable in 74.2% by the phages of SLOPEK *et al.* [3]. The most frequent *Klebsiella* phage types were VI.A1, I.A1, I.B1, II.A1 and IV.A1, according to SLOPEK *et al.* [3]. The Hungarian strains isolated from a hospital and from outpatients in 1969 belonged to 23 phage types; the most frequent types were I.B1 and II.A1. In the present study the frequency of phage types was analysed according to species: phage type II.A1 was the most frequent among *K. aerogenes* and *K. atlantae* strains, and other 9 and 5 phage types were demonstrated. The most heterogeneous phage type distribution was observed among the strains isolated from urine. The spread of phage type II.A1 might be accidental, but was probably due to selection by the applied antibiotics, because the number of multiresistant strains belonging to that phage type was rather high.

K. ozaenae strains were typable in 23.7% by phages in our material. According to SLOPEK *et al.* [3] and RENNIE *et al.* [10], *K. ozaenae* strains were not or only exceptionally typable by type phages. We suggested that our strains contained other K antigens which did not affect their phage sensitivity. RENNIE *et al.* [10] observed that untypable *K. ozaenae* strains were sensitive to phages.

Our studies demonstrated that *Klebsiella* phage typing was suitable for epidemiological purposes and to substitute the complicated sero- and bio-typing, though the "not typable" results limited the efficiency of the method. A further development of the method requires the isolation and adaptation of new phages.

Diagnostic phage C14 lysed 64% of the strains belonging to *Enterobacter*, but no *Klebsiella* strain was lysed by this phage. Lysis by phage C14, accordingly, is suggestive of *Enterobacter*. Identification by biochemical tests can be completed by the use of phage C14, in the case of positive reaction. We suggest to use sensitivity to phage C14 as a diagnostic method to identify the genera *Klebsiella* and *Enterobacter*.

Multiresistance was demonstrated in 36.3% among *Klebsiella* and in 16.5% among *Enterobacter* strains.

The high frequency of multiresistance was due to *K. rhinoscleromatis*, *K. edwardsii* and *K. pneumoniae* strains. Among *Enterobacter* strains, in the species *E. liquefaciens* the frequency of multiresistance was extremely high.

MØLLER *et al.* [11] examined the antibiotic sensitivity of *Klebsiella* and *Enterobacter* strains isolated from patients. Strains sensitive to the antibiotics tested were not found among the *Klebsiella* strains, while 10% of the strains of genus *Enterobacter* were sensitive to antibiotics. *Klebsiella* and *Enterobacter* strains were resistant to one or two antibiotics in 74% and 66%, respectively; they were multiresistant in 26% and 24%, respectively.

Comparing the data on antibiotic resistance from a Danish hospital and from our examinations, the number of resistant strains was higher in our material. The difference was striking in the case of tetracycline and chloramphenicol, as *Klebsiella* strains isolated in Denmark were resistant to tetracycline in 5%, those tested by us in 31.3%; chloramphenicol resistance was 6% and 31.3%, respectively. Resistance to kanamycin and nalidixic acid was five and two times higher in the Hungarian strains, respectively. The differences observed in the resistance of the strains derived from the two countries were probably due to the different use of antibiotics. In our material, four *Klebsiella* strains were resistant to polymyxin-B. According to the 1977 Report of the Public Health Stations no polymyxin-B resistant strain was isolated [12].

TKACZOWA *et al.* [13] found drug-resistant strains among *K. aerogenes* in 47.7%, *K. rhinoscleromatis* in 32.3%, *K. edwardsii* in 29.8%, *K. pneumoniae* in 28.6%, *K. atlantae* in 24.1% and *K. ozaenae* in 9.9%.

Examining the strains derived from different hospitals according to the type of resistance (mono, double and multiple resistance) different types were found, though the strains had been isolated in the same country. *Klebsiella* strains isolated from a urological ward were multiresistant in 83.2% and 12% were multiresistant of those isolated from infant and premature infant wards of a county hospital. The occurrence of multiresistance among the *Enterobacter* strains varied significantly, from 61.9% to 11.0%. The differences observed in the frequency of multiresistant strains were probably due to differences of the antibiotic therapy.

The strains resistant only to ampicillin were divided into 6 groups according to their cephaloridine and cephalothin resistance [14]. Out of the 36 ampicillin resistant strains those which belonged to group 1 were resistant to the three drugs. Strains of group 2 were partially resistant to ampicillin, apparently by a non-enzymatic mechanism, and they were fully resistant to cephaloridine and cephalothin; according to the opinion of GREENWOOD and O'GRADY [14] these strains were weak β -lactamase producers. Group 3 strains were partially resistant to cephalothin and fully resistant to the two other drugs. Groups 4 and 5 strains were partially resistant to ampicillin, fully resistant to cephaloridine and sensitive or partially sensitive to cephalothin. Group 6 strains were sensitive to cephalothin only. The above-mentioned authors supposed that among the representatives of this group, cephalothin was more stable in the presence of β -lactamase.

There was a contradiction in the correlation between antibiotic resistance and phage resistance in *Klebsiella* and *Enterobacter* strains. Antibiotic sensitive *Klebsiella* strains were typable by phages in 10.7%, multiresistant strains in 29.1%. Monoresistant *Enterobacter* strains were sensitive to phage in 74.4%, multiresistant ones in 35.3%. A correlation was observed between typability and multiresistance of *Klebsiella* strains: multiple resistant strains were typable in 29.1%, and not typable in 70.9%. Considering these results we suggested that resistance to phages in *Enterobacter* strains was connected with the presence of R-plasmids determining multiple resistance. The conclusion was influenced by the fact that only five strains were examined in the antibiotic sensitive group.

Our studies demonstrated that multiresistance was common among *Klebsiella* and *Enterobacter* strains derived from hospitals; it varied from 14.5% to 66% and from 16.5% to 46.0%, respectively, according to the source of isolation. Multiresistance was significantly lower among the hospital strains isolated from nose, throat and faecal samples than those isolated in high number from urine from a hospital ward. The selective effect of the antibiotics used for prevention in urinary tract infections offered an explanation to these findings.

COOKE *et al.* [7] demonstrated *Klebsiella* strains in 16% among coliforms isolated from a general hospital ward during a 22 month period. They studied the connection between species, serotype, colicin type and infection and found that in 50% of the patients the type of the strains isolated from wound infections was the same as that of the strains carried in the intestine.

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SURFACE IMMUNOGLOBULINS OF TONSIL LYMPHOCYTES

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Human tonsillar lymphocytes bound ^{14}C -labelled homologous IgG, IgA and IgM at 5 °C and released them at 35 °C. B cells were found to be more active in this type of Ig binding. The amount of lymphocytes which could be labelled with fluoresceinated anti-human Ig-s decreased during preincubation of the cells at 35 °C. Intrinsic Ig-s (determinants of lymphocytes) were demonstrated by immunofluorescent staining preceded by preincubation of the cells at 35 °C for 60 min. The ratios of IgG, IgA and IgM bearing cells in the human tonsillar lymphocyte population were 16.0, 8.0 and 7.4% respectively. The weak binding of Ig-s to the surface of lymphocytes is discussed with regard to the demonstration of surface Ig determinants of lymphocytes.

Characterization of the tonsillar lymphocyte population is necessary for the elucidation of the particular immunological function of the tonsils. The tonsils have been shown to contain 42–55% T and 43–54% B lymphocytes [1, 2]. Further classification of B cells on the basis of surface immunoglobulins has been undertaken by several authors by rosette formation and immunofluorescent methods [1, 3–7]. Their data show considerable variations.

The percentage of the surface Ig bearing cells has been found to be 39.5% [3] and 54% [6] by fluorescent staining with polivalent anti-Ig.

Surface IgG has been demonstrated by immunofluorescence in about 40% [3] and 6% [5] of the Ig-bearing cells. Individual variations were observed, but these could not account for the wide differences between the results. Earlier observations have indicated the dynamic state of membrane-bound proteins of lymphocytes. The half-life of IgM on the surface of tonsillar cells is about 4 h, resulting in a continuous release of IgM into the medium [8].

A considerable part of the surface Ig demonstrable at 4 °C has been shown to elute at 37 °C [9–11]. Shedding of membrane components occurred in consequence of the 4 to 37 °C temperature shift [12] and this was used for the isolation of antigen receptors [13], Fc, SRBC and C_3 receptors [14, 15]. Shedding of membrane components is only partly dependent on active cellular metabolism [12, 15].

These temperature-related effects may have a marked influence on the fluorescent staining of lymphocytes with heterologous anti Ig-s. (In earlier studies, fluorescent staining has been carried out at 4 °C.) Adsorption or binding of homologous Ig-s to the Fc receptors of cells may also alter the staining properties of lymphocytes. The present study has been undertaken to assess

and possibly to eliminate the influence of the above factors, and to determine by immunofluorescent staining at 35 °C the amount of IgG, IgA and IgM bearing lymphocytes in the tonsillar cell population.

Materials and methods

Isolation of lymphocytes. Clinically normal palatine tonsils of 2 to 5-year-old children were used immediately after tonsillectomy. The tonsils were cut into slices with razor blades. The slices were suspended in PBS (phosphate buffered saline, pH 7.4) solution, and the lymphocytes were removed from the tissue by magnetic stirring at 4 °C for 15 min. The suspension was filtered through a double layer of gauze and centrifuged at 300 g at 4 °C for 10 min [16]. The sedimented cells were suspended in Parker TC 199 medium diluted with one volume PBS. Phagocytic cells were removed by carbonyl iron [17], thereafter the cell suspension contained about 99% lymphocytes. The E-rosette test performed according to JONDAL *et al.* [18] demonstrated about 31–38% T lymphocytes.

Tonsillar lymphocytes were fractionated into T and B cells by adsorption to nylon wool [2]. The ratio of E-rosette forming lymphocytes was 2–5% in the B cell fraction, and 87–91% in the T cell fraction. The difference between these cell fractions was demonstrated by measuring the effect of PHA on [³H]-thymidine incorporation in cultures of T or B cells (Table I). Large stimulation of DNA synthesis was found with T cells only.

Table I

Influence of PHA on [³H] thymidine incorporation by B and T lymphocytes

Tonsillar lymphocytes	Expt.	Control dpm/10 ⁶ cells	PHA/10 µg/ml dpm/10 ⁶ cells	PHA control
Unseparated	1	1800	3800	2.1
	2	1200	3600	2.2
T cells	1	800	7500	9.3
	2	500	6000	12.0
B cells	1	700	1000	1.4
	2	600	900	1.5

Tonsillar cells (2×10^6 /ml) were incubated in Eagles MEM in the presence of 1.1×10^2 kBq/ml [³H] thymidine, added 1 h before the end of the 72 h incubation period. Radioactivity of the trichloroacetic acid precipitated DNA was counted by liquid scintillation in toluene based cocktail, and expressed as dpm/10⁶ cells

Conjugation of fluorescein isothiocyanate to anti-human IgA, IgG and IgM. Monospecific anti-human immunoglobulin sera (National Institute for Serobacteriological Production and Research, Human, Hungary) were purified by solid-phase absorption. IgG fractions were isolated by DEAE Sephadex A 50 chromatography and labelled with fluorescein isothiocyanate isomer I (FITC, Spofa). The free dye was separated from the conjugates by gel filtration on Bio Gel P-10. The lyophilized conjugates were dissolved in diluted Parker medium before use. Characteristics [19, 20] of the working dilutions of the FITC labelled anti-IgG, IgA and IgM preparations are shown in Table II.

Preparation of ¹⁴C-labelled human IgA, IgG and IgM. Freshly prepared tonsillar lymphocytes (2×10^6 /ml) were incubated in valine-free Hanks' medium supplemented with 0.1 mmole/l of each of 18 amino acids, 3 mmole/l glutamine, 10 mmole/l glucose, 100 U/ml penicillin,

Table II

Properties of the working dilutions of fluoresceinated antiglobulin preparations

	RAHu/IgG	ShAHu/IgA	ShAHu/IgM
Degree of labelling expressed as molecular ratio of Ig and dye	5.11	2.72	1.28
Concentration of Ig, g/litre	8.0	16.0	6.7
Precipitating antibody, g/litre	0.5	0.5	0.5

100 $\mu\text{g/ml}$ streptomycin, and 92 kBq/ml of [^{14}C]-valine (sp. act. 6.47×10^6 kBq/mole, UVVR, Prague) at 37 °C for 3 h. Then cells were frozen and thawed twice and homogenized in PBS. After centrifugation at 5000g for 10 min at 0 °C labelled Ig-s were isolated from the supernatant by immuno-adsorption as follows: Ig fractions of goat antisera to human IgG, IgA or IgM (Hyland, England) were covalently attached to AH-Sepharose 4 B (Pharmacia, Sweden) by the method of CAMBIASO *et al.* [21]; 12–18 mg of goat immunoglobulin was immobilized on one ml of gel. Aliquots of the supernatant of homogenized lymphocytes were incubated with the immuno-adsorbents at 37 °C for 1 h, then non-bound molecules were removed by washing with PBS. The immunocomplexes were dissociated by treatment with 0.35 M glycine-HCl buffer, pH 2.4, containing 0.1 M glucose. The eluate containing the Ig-s was neutralized by Na_2CO_3 . Radioactivity of the immunoglobulins thus obtained was determined by liquid scintillation using a toluene-based cocktail containing 25% Triton X-100. The specific radioactivity of the resulting preparations were 1.5×10^4 dpm/ μg IgG, 1×10^4 dpm/ μg IgM, and 3.6×10^4 dpm/ μg IgA.

Fluorescent staining of tonsillar lymphocytes. Freshly prepared lymphocytes were washed twice at 4 °C, then suspended in 2 ml diluted Parker medium (10^7 cells/ml). The cells were preincubated before staining; the duration and temperature of preincubation was varied as described. Thereafter the cells (2×10^7) were sedimented and suspended in 0.2 ml diluted Parker medium, and 0.05 ml of the working dilution of FITC labelled anti-human Ig was added; temperature and duration of the stainings were varied, too.

Stained cells were washed twice with diluted Parker medium at the temperature of staining, then suspended in 0.1 ml of a mixture of 4 volumes of glycerol and one volume of 0.5 M phosphate buffer, pH 8.0. Stained cells were counted in a Gilbert-Silbert fluorescence microscope equipped with a dark field condenser and broad band interference exciting filter. In each preparation 500 cells were counted and the ratio of stained cells was expressed in per cents of the total. The intensity of fluorescence of the stained cells was evaluated semiquantitatively as faint (+), medium (++) and intense (+++).

Results

Binding to lymphocytes of ^{14}C -labelled immunoglobulins. ^{14}C -labelled IgG, IgA and IgM were prepared from tonsillar lymphocytes as described in Methods. Separated tonsillar T and B lymphocytes as well as unseparated lymphocytes were incubated with the labelled Ig fractions at 5 °C for 60 min (Table III). Thereafter the cells were washed with diluted Parker medium at 5 °C and the activity bound by the cells was determined. The unseparated lymphocytes bound about 60–70% of the labelled Ig-s. More Ig was bound to B cells than to T cells. Labelled Ig bound to the cells at 5 °C was released quantitatively from the lymphocytes on incubation at 35 °C for 60 min. The experiment indicated a temperature dependent association and dissociation between lympho-

Table III

Binding of homologous, ^{14}C -labelled Ig-s by T and B lymphocytes at 5 °C

Cell	Ig bound to cells, per cent of total Ig added		
	IgA	IgG	IgM
Unseparated lymphocytes	60	60	74
B cells	85	87	79
T cells	12	10	20

Labelled Ig ($0.5\ \mu\text{g}$) was incubated with 10^8 lymphocytes in 1 ml of diluted Parker medium at 5 °C for 60 min, then the cells were washed with the same medium at 5 °C. Radioactivity bound to the cells was determined by liquid scintillations

cytes and immunoglobulins, which may have influenced the labelling of lymphocytes with fluoresceinated anti-Ig-s.

Effect of the duration and temperature of preincubation and staining on the fluorescent labelling of lymphocytes with anti-IgG conjugate. Lymphocytes were preincubated for 60 min and stained for 60 min at the same temperature (Fig. 1). The percentage of the labelled cells decreased markedly on increasing the temperature of the treatment. The intensity of staining increased with increasing temperature. Duration of the preincubation of cells at 35 °C also influenced the amount of labelled lymphocytes (Fig. 2, staining at 35 °C for 60 min). The percentage of labelled lymphocytes decreased with the increase of preincubation time. No change was observed in the intensity of staining.

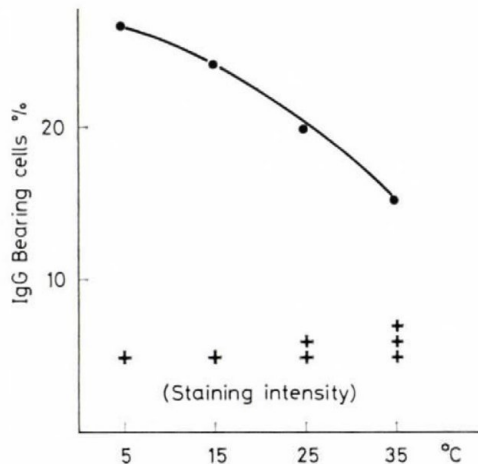


Fig. 1. Staining intensity and the ratio of IgG-bearing lymphocytes on increasing the preincubation temperature (60 min), and staining (60 min)

After preincubation at 35 °C for 60 min, the duration of staining at 35 °C did not alter the number of labelled lymphocytes (Fig. 3). The staining intensity increased, however, with the increase of the staining time.

According to the above experiments, the lowest number of lymphocytes labelled with fluoresceinated anti-IgG was obtained after a preincubation of 60 min at 35 °C. Intensive fluorescent labelling can be achieved by staining the cells at 35 °C for 60 min.

Demonstration of surface IgG, IgA and IgM on tonsillar lymphocytes. Tonsillar lymphocytes were preincubated at 5 °C or 35 °C for 60 min, then

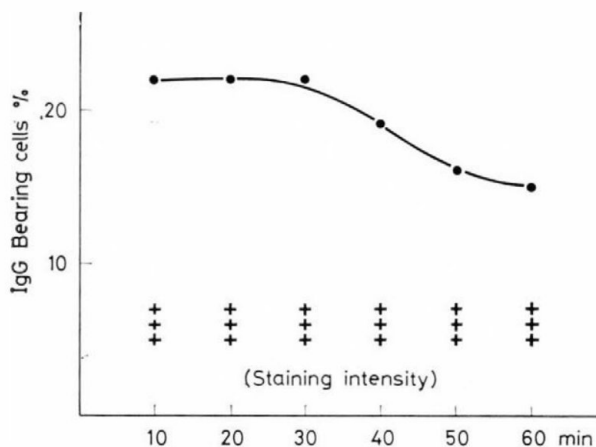


Fig. 2. Ratio of IgG-bearing lymphocytes with preincubation time at 35 °C and staining at 35 °C for 60 min

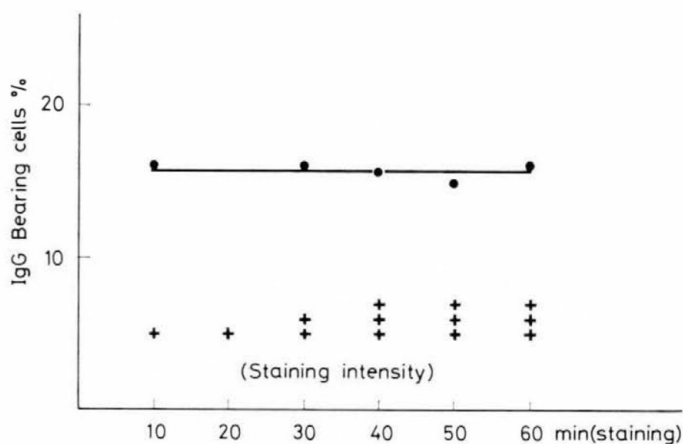


Fig. 3. Staining intensity of lymphocytes with increasing staining time. Preincubation at 35 °C for 60 min, staining at 35 °C

Table IV
Demonstration of surface IgG, IgA and IgM on tonsillar lymphocytes

Preincubation temperature	Percentage of cells labelled with fluoresceinated antiglobulin specific for		
	IgG	IgA	IgM
5 °C	29.0	15.0	22.0
	± 1.20	± 0.80	± 1.12
35 °C	16.0	8.0	7.4
	± 1.03	± 0.69	± 0.99

Tonsillar lymphocytes were preincubated at 5 °C or 35 °C for 60 min, than stained with FITC-labelled anti-Ig-s at 35 °C for 60 min as described in Methods. Data are expressed as mean $\pm$ SD, n = 11

stained with FITC-labelled anti-human IgG, IgA and IgM at 35 °C for 60 min. The results in Table IV show that the temperature of preincubation considerably influenced the staining; the most marked difference (7.4% vs. 22%) was found in the number of anti-IgM-labelled cells.

Discussion

The amount of surface Ig of tonsillar lymphocytes can be increased by attachment of homologous Ig-s at 5 °C, as demonstrated by the experiment with ^{14}C -labelled IgG, IgA and IgM. B cells bound more Ig than did T cells. Ig-s bound in the cold were released quantitatively at 35 °C. It is assumed that Fc receptors and/or specific receptors for Ig-s [5] are responsible for this type of binding, although some aspecific attachment cannot be excluded. Release of surface Ig-s was indicated by the temperature dependent decrease of the amount of labelled cells on fluorescent staining with heterologous anti-Ig-s. The decrease of the amount of surface Ig at 35 °C in 30–40 min may be interpreted as a release of molecules bound during preparation at low temperature. A similar, rapid release of surface Ig has been observed by several authors [9–12]. KUMAGAI *et al.* [9] considered the cell-bound, labile Ig as serum IgG attached to Fc receptors. A subpopulation of human peripheral lymphocytes capable of reversible binding of homologous Ig-s has been suggested by LOBO *et al.* [10]. The initial rapid phase of the elution of membrane Ig-s at 37 °C was not influenced by metabolic poisons [12]. The nature of the labile binding of Ig to the membrane is still unexplained. Nevertheless, the presence of surface Ig, which is not the product of the particular lymphocyte, has to be considered in the examination of surface markers.

In view of these properties of surface Ig-s, we assume that endogenous surface Ig determinants of B cells can be demonstrated selectively after pre-incubation of lymphocytes at 37 °C. Fluorescent staining of cells at low temperatures results in a labelling of cells with labile surface Ig, too (Fig. 1), and this may explain the high percentage of Ig bearing cells found in earlier studies. It is concluded that in the tonsillar lymphocyte population the percentage of cells with IgG, IgA and IgM surface determinants is 16.0, 8.0 and 7.4%, respectively.

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EFFECT OF *BORDETELLA PERTUSSIS* VACCINE ON NEONATALLY THYMECTOMIZED MICE

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Lymphocytosis and splenic hypertrophy, the characteristic effects of *Bordetella pertussis* vaccine in mice with intact lymphoid system, were observed to develop in neonatally thymectomized 4-week-old CFLP mice. Splenomegaly was associated mainly with the role of granulocytes.

Previous studies have indicated that neonatal thymectomy of mice retards the development of lymphoid organs and produces grave lymphoid atrophy, as compared to sham-thymectomized control animals [1, 2]. It is also known that *Bordetella pertussis* vaccine causes lymphocytosis in peripheral blood, and splenomegaly [3, 4]. It was therefore studied how neonatally thymectomized mice with atrophic lymphoid organs respond to a treatment with pertussis vaccine.

Materials and methods

Experimental animals. The experiments were carried out on 4-week-old CFLP mice of both sexes.

Neonatal thymectomy. The thymus was removed according to MILLER's technique, 24 h after birth [5]. At the same time sham-thymectomy was performed on mice of the same litters.

Treatment with pertussis vaccine. The vaccine used contained 30×10^9 killed bacteria/ml in physiological saline (Institute for Serobacteriological Production and Research Human, Budapest). The mice received single intraperitoneal (i.p.) injections of 0.3 ml pertussis vaccine (9×10^9 bacteria/mouse). The control mice received the same quantity of physiological saline i.p.

Examination of the lymphoid system. White blood cell (WBC) count and absolute number of lymphocytes were determined in blood taken from the caudal vein under standardized conditions. Mean relative spleen weight and spleen index of the sacrificed animals were calculated as follows:

$$\text{Relative spleen weight} = \frac{\text{spleen weight (mg)}}{\text{body weight (g)}}$$

$$\text{Spleen index} = \frac{\text{mean relative spleen weight in the experimental group}}{\text{mean relative spleen weight in the control group}}$$

Histological examination. The spleen of the sacrificed mice was fixed in formalin and sections were stained with haematoxylin-eosin.

Statistical evaluation. For statistical evaluation Student's two sample *t* test was applied. The significance level was at $p = 0.05$.

Results

A total of 30 neonatally thymectomized and 40 neonatally sham-thymectomized mice were used. Half of both groups were treated with pertussis vaccine at the age of 4 weeks (T⁻-P and T⁺-P groups). The rest received physiological saline (T⁻ and T⁺ groups). Table I shows the groups, the number of mice, the operations and the treatment.

Table I
Groups, number of mice, operations and treatment

Group	No. of animals	Neonatal operation	Treatment i.p.
T ⁻ -P	15	thymectomy	pertussis vaccine
T ⁺ -P	20	sham-thymectomy	pertussis vaccine
T ⁻	15	thymectomy	saline
T ⁺	20	sham-thymectomy	saline

On the 5th day following pertussis vaccine administration when the lymphocytosis and spleen hypertrophy were marked [3, 4, 6] WBC count and absolute number of lymphocytes were determined; then the animals were sacrificed and the relative spleen weight and spleen index were calculated.

The success of neonatal thymectomy was controlled by gross examination. Only successfully (completely) thymectomized mice have been included in the T⁻ and T⁻-P groups.

Table II summarizes the mean WBC count and the absolute number of lymphocytes. As indicated, the WBC count and the absolute number of

Table II
White blood cell (WBC) count and absolute values for lymphocytes in peripheral blood 5 days after injection of B. pertussis vaccine

Group	WBC count	Significance	Absolute number of lymphocytes	Significance
T ⁺	7 800 ± 760	p < 0.001	6 000 ± 1 045	p < 0.001
T ⁻	4 900 ± 1 344		2 200 ± 668	
T ⁺ -P	59 200 ± 12 600	p > 0.7	31 900 ± 9 710	p > 0.5
T ⁻ -P	57 800 ± 19 000		22 500 ± 9 841	

lymphocytes were significantly lower in the case of neonatally thymectomized mice (T^-) than were the corresponding values obtained in group T^+ .

Pertussis vaccine raised the WBC count almost to the same level in both the T^-P and the T^+P groups without any significant difference. Nevertheless, the increase in WBC count was 11-fold in the T^-P group, while that of the T^+P group was 7.5-fold as compared to the corresponding values obtained in groups T^- and T^+ . It also caused a considerable increase in the lymphocyte count in both groups. Comparison of the lymphocyte values in groups T^- and T^+ shows that the absolute number of lymphocytes increased only 5-fold in the T^+P group and 10-fold in the T^-P group.

Figure 1 represents the relative spleen weight and spleen index as calculated after sacrifice.

The relative spleen weight was significantly lower in the neonatally thymectomized mice (T^-) than in the sham-thymectomized mice (T^+). The pertussis vaccine caused splenomegaly in both the thymectomized and the sham-thymectomized groups (T^-P and T^+P); there were no significant differences between the two treated groups. Considering the values obtained in the untreated groups, the splenomegaly was more marked in the T^-P than in the T^+P group, i.e. just as with the absolute lymphocyte count, the increase in relative spleen weight was greater in the thymectomized group. The spleen index of group T^-P was nearly 4, while that of group T^+P was 2.

Histological examination revealed a normal spleen structure in the T^+ group, while it showed atrophy characterized by cell depletion and consider-

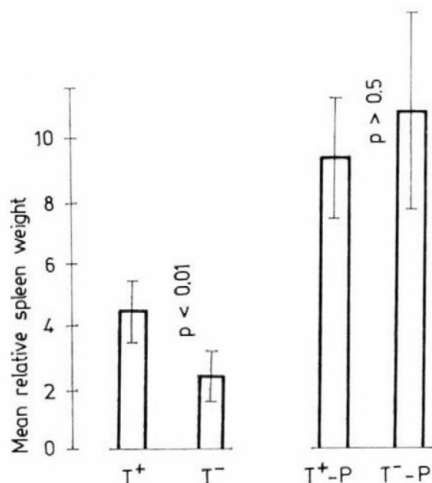


Fig. 1. Data of the lymphoid organs. Spleen index:

$$\frac{T^+P}{T^+} = 2.0 \quad \frac{T^-P}{T^-} = 3.9$$

ably less and smaller follicles in group T⁻. The pertussis vaccine caused an increase in cell count, hyperplasia, enlarged follicles and germinal centres in the spleen of the T⁺-P mice.

There was a diffuse increase of cells in the spleen of the thymectomized and vaccinated mice and, similarly as in the T⁻ group, the normal spleen structure was hardly recognizable. The follicles remained small and, in contrast with the expressed infiltration with lymphocytes of the spleen of T⁺-P mice and the scarcity of granulocytes in the T⁻-P mice, the pulp showed a diffuse increase in granulocytes.

Discussion

By the time the neonatally thymectomized mice were 4 weeks old, the consequences of neonatal thymectomy were accentuated: the absolute number of lymphocytes was low, weight measurements and histological examination revealed spleen atrophy.

The pertussis vaccine produced excessive lymphocytosis and splenomegaly due to hypertrophy and hyperplasia in the T⁺ mice. These findings agree with earlier reports and our own previous experiments.

The vaccine also affected the atrophic lymphoid system of thymectomized (T⁻) mice. Their sensitivity to the vaccine was higher than that of the sham-thymectomized mice. As to the type of cell which had a role in the increase in the WBC count, the vaccine caused granulocytosis in addition to lymphocytosis and the hyperplasia of the spleen was caused mainly by the granulocytes. This is supported by the observation that following pertussis vaccine treatment in neonatally thymectomized inbred mice, the increase in WBC count was due to a significant increase in the number of polymorphonuclear cells and to a slight increase in the number of lymphocytes. Besides, a relative myeloid hyperplasia was found in the spleen [7]. Similarly, the vaccine led to an increase of granulocytes in the peripheral blood of congenitally athymic nude mice [8]. Earlier we observed the appearance of granulocytes on the choriomeninges in runted mice suffering a GvH reaction after intracerebral infection with LCM virus [9].

These results indicate that an intervention which causes lymphocytosis in mice with an intact immune system, leads to granulocytosis in mice with impaired immune system and stresses the importance of granulocytes in this process.

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EFFECT OF METHICILLIN ON THE FATTY ACID COMPOSITION OF PHOSPHOLIPIDS IN METHICILLIN SENSITIVE *STAPHYLOCOCCUS* *AUREUS**

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The effect of two concentrations of methicillin on the distribution of fatty acid (FA) in the individual phospholipids of the middle-log phase cultures of *Staphylococcus aureus* 5814S sensitive to methicillin was studied during a period of 2 h. In the presence of $\frac{1}{2} \times$ minimum inhibitory concentration (MIC) of methicillin ($= 1 \mu\text{g/ml}$) the ratio of unsaturated fatty acid (UFA) + branched fatty acid (BFA) to saturated fatty acid (SFA) in the phosphatidic acid (PA) and in the C_{55} -isoprenylphosphate (C_{55} -IP) became significantly higher due mainly to the accumulation of n-C12: 3, ai-C17: 0, and ai-C19: 0, and to the fall of n-C14: 0, n-C16: 0, and n-C20: 0 in the PA, and to the 3-fold increase in n-C12: 3 and a 2-fold decrease in the SFAs of the C_{55} -IP. In contrast, the ratio of UFA + BFA to SFA was smaller in the phosphatidylglycerol (PG) and lysylphosphatidylglycerol (LPG) than that of the control. The reduction was attributed to a loss of ai-C15: 0 and a gain of n-C20: 0 in the PG, and to the decrease in ai-C17: 0 and to the increase in n-C20: 0 in the LPG. On the whole, the fluidity of the membrane phospholipids decreased. In the presence of $1 \times \text{MIC}$ of methicillin ($= 2 \mu\text{g/ml}$) the ratio of UFA + BFA to SFA became higher in the PA due mainly to the prevention of the release of n-C18: 1. In all the other phospholipids the ratio of UFA + BFA to SFA was smaller than in the control of the same age. The decrease in the ratios ranged in the order of C_{55} -IP, PG, diphosphatidylglycerol (DPG) and LPG. The changes were attributed to the decrease in the proportion of n-C12: 3 and ai-C15: 0 or ai-C17, and to the increase in n-C20: 0, except DPG. Thus the membranes in methicillin treated cocci were significantly less fluid than in the controls.

It has been reported that the lipid content and composition as well as the FA distribution in lipids of various bacterium species change under various cultural conditions both *in vitro* and *in vivo* [1–13]. On the other hand, a number of antibacterial agents has been shown to influence the synthesis and/or composition of lipids in bacteria, although only few antibacterial agents were shown direct special inhibitors of a certain step of lipid biosynthesis [14–25].

In previous studies we have found that methicillin at a concentration of $\frac{1}{2} \times \text{MIC}$ ($= 1 \mu\text{g/ml}$) causes a significant increase in the amount of all the phospholipids in the exponentially growing cultures of the methicillin-sensitive *Staphylococcus aureus* 5814S during a 2-h treatment. In contrast, the addition of $1 \times \text{MIC}$ of methicillin ($= 2 \mu\text{g/ml}$) to the cultures resulted in a significant

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decrease in the acid phospholipids of the cocci. The content of alkaline LPG in the cells of the cultures is, however, enhanced in the 1st hour and then is reduced to the control value by the 2nd hour of methicillin exposure. From these results it has been concluded that methicillin influences the phospholipid synthesis and composition in *S. aureus* and may contribute to the survival or the death of the cocci under the effect of different concentrations of methicillin [20, 26].

The FA composition of phospholipids was found to govern the permeability rate of non-electrolytes through the biological membranes as well as the rate of the facilitated diffusion process [15].

In this paper detailed evidence are given of the changes in the FA distribution of individual phospholipids in logarithmically growing cultures of *S. aureus* 5814S under the effect of methicillin.

Materials and methods

Materials. All chemicals were of the best commercial grade, essentially as described previously [26, 27].

Organism and growth conditions. The methicillin-sensitive *S. aureus* 5814S strain [28, 29] was used for the experiments. The strain was maintained on slants of nutrient agar at room temperature. The lipid-free medium used at pH 7.2 in the experiments was as described by VÁCZI et al. [30]. Methicillin was purchased from Chinoïn, Budapest.

For the experiments 7 litres of broth in a 10-litre retort were inoculated with an overnight broth culture of the strain, so that $1.2-1.5 \times 10^7$ organisms per ml served as starting inocula. Cultivation was carried out as described previously [26]. When the middle log-phase had been reached, $1 \mu\text{g/ml}$ ($= \frac{1}{2} \times \text{MIC}$) or $2 \mu\text{g/ml}$ ($= 1 \times \text{MIC}$) of methicillin was added to the culture. This time was indicated as 0 h. Further incubation lasted for 2 h at 37°C . At 0, 1 and 2 h of methicillin exposure, samples were obtained to determine the viable counts, the dry weight and for the lipid extraction [26, 27]. Each type of experiment was repeated at least 4 times.

Lipid extraction and separation of phospholipids. All the methods utilized for the extraction, purification, separation and identification of lipids were described previously [26, 27]. Phospholipids for FA analysis were again purified on Sephadex G-25 fine after eluting them from the Silica gel G of the chromatoplates. In order to obtain a better separation of the DPG from the polar pigments it was rechromatographed in Silica gel G plates in one dimension, using chloroform-acetone-methanol-acetic acid-water 65:35:11:4:1.5, as developing system.

Gas chromatography of fatty acids. Methylation of the phospholipid FAs was performed with 5% HCl in methanol at 80°C for 2 h. The other methods applied for the analysis of FA methyl esters were essentially the same as described by CHRISTIE [31] and BIACS [32]. A mixture of FA methyl esters was resolved by capillary column gas chromatography using a polar column coated with 10% of EGSS-X in a Chromatron 18.3 CHF gas chromatograph. Isotherm temperatures were 175°C and 190°C . The relative retention times of all the FAs were confirmed with available standards of Packard KIT and with wool-wax of known composition (for iso- and anteisoacids).

Results

Polar lipid content. Figure 1 shows the effect of two concentrations of methicillin on the polar lipid (PL) content of log-phase cocci during a period of 2 h.

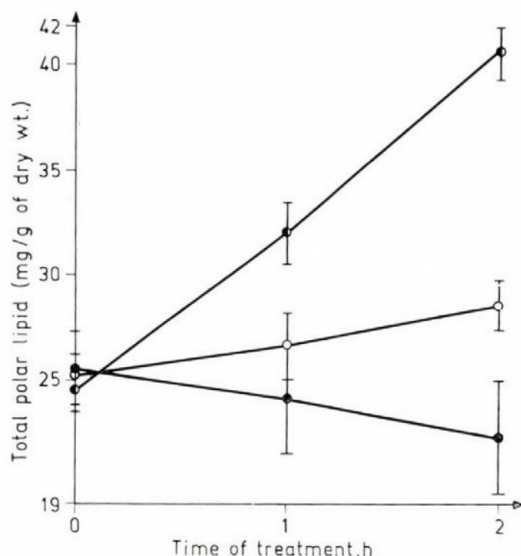


Fig. 1. Effect of methicillin on the polar lipid (PL) content of *Staphylococcus aureus* 5814S growing exponentially. ○—○ control (4); ◐—◐ 1 μ g methicillin per ml = $\frac{1}{2} \times \text{MIC}$ (5); ●—● 2 μ g methicillin per ml = $1 \times \text{MIC}$ (5). Figures in brackets indicate the number of experiments.

The PL level rose in the control cells with the proceeding of the log-phase. Addition of $\frac{1}{2} \times \text{MIC}$ of methicillin to the cultures caused in the PL content of the cells a 1.2-fold increase in the 1st hour and a 1.42-fold increase in the 2nd hour of the treatment as compared to controls of the same age. In contrast, under the influence of $1 \times \text{MIC}$ of methicillin the PL content of the cocci fell below the control value. After growth with 2 μ g/ml of methicillin for 2 h, the PL level was lower than it was at 0 time and 0.78-fold of the control of the corresponding age.

The bulk of PL in the membranes consists of phospholipids. The structure and composition of the FA portions of the phospholipids are known to influence the cell wall synthesizing and hydrolyzing enzymes associated with the membranes.

Fatty acid distribution of phosphatidic acid. The effect of two concentrations of methicillin on the distribution of FA in the PA in logarithmically growing cells of *S. aureus* 5814S is shown in Table I.

The predominant FAs in the PA of 0-time cultures were ai-C15:0, n-C20:0, and i-C26:0 acids. In the PA of the untreated cocci the proportion of n-C12:3, n-C14:0, ai-C15:0, ai-C19:0, and n-C20:0 increased considerably, while that of n-C16:0, n-C16:1, ai-C17:0, n-C18:1, n-C19:0, and i-C26:0 decreased considerably during a 2-h period of the middle log-phase. Consequently, although ai-C15:0 and n-C20:0 remained the two main com-

Table I

Effect of methicillin on the fatty acid composition of phosphatidic acid in exponentially growing *S. aureus*

Fatty acid	Per cent fatty acid in PA of cells in cultures			
	of 0-time control	of 2-h control	treated with	
			$\frac{1}{2} \times \text{MIC}$	$1 \times \text{MIC}$
			of methicillin for 2 h	
n-C12: 3	2.6	5.6	8.2	2.0
n-C12: 0	—	—	1.2	0.8
n-C13: 0				
i-C14: 0	—	0.4	—	—
n-C14: 0	trace	2.7	0.7	1.2
ai-C15: 0	24.3	46.0	48.1	26.2
i-C16: 0	—	0.7	0.9	1.6
n-C16: 0	4.3	1.1	1.8	5.2
n-C16: 1	8.7	—	—	—
ai-C17: 0	5.8	1.8	7.6	13.2
i-C18: 0	—	0.1	0.2	0.9
n-C18: 0	7.7	6.2	4.5	7.5
n-C18: 1	6.7	—	—	5.8
ai-C19: 0	—	2.3	3.6	7.0
n-C19: 0	5.3	2.7	3.6	5.3
n-C20: 0	16.8	30.2	19.6	17.3
n-C21: 0	2.4	0.2	—	1.9
i-C22: 0	3.6	—	—	1.6
i-C26: 0	11.8	—	—	2.5
Unsaturated	18.0	5.6	8.2	7.8
Branched	45.5	51.3	60.4	53.0
Saturated	36.5	43.1	31.4	39.2
Ratio of UFA + BFA to SFA	1.74	1.32	2.18	1.55

ponents, the fluidity of PA was decreased, since the ratio of UFA + BFA to SFA became smaller.

Addition of $\frac{1}{2} \times \text{MIC}$ of methicillin to the cultures resulted in a pronounced enhancement of the proportion of n-C12: 3, ai-C15: 0, and ai-C19: 0, whereas in a substantial fall in n-C16: 0, n-C16: 1, and n-C18: 1, as compared to the 0-time control value. On the other hand, in the presence of $\frac{1}{2} \times \text{MIC}$ of methicillin the proportion of n-C14: 0 and n-C20: 0 failed to increase and that of ai-C17: 0 failed to decrease to such an extent as in the control. Thus, the

branched fatty acids were incorporated into the PA of the treated cocci in a greater amount and the unsaturated fatty acids were released in a smaller amount than the saturated ones, consequently the ratio of UFA + BFA to SFA became significantly higher than in the untreated cocci, leading to an increase in the fluidity of the PA in cocci exposed to $\frac{1}{2} \times \text{MIC}$ of methicillin.

In contrast, when $1 \times \text{MIC}$ of methicillin was added to the cultures, the FA distribution of the PA persisted nearly at the 0-time distribution. During a 2-h treatment none of the FAs changed to such an extent as in the control. There was an increase in ai-C17:0 and ai-C19:0, while a decrease in n-C16:1 and i-C26:0, compared with the 0-time values. On the whole, the loss of unsaturated fatty acids was not accompanied by a gain of branched fatty acids, and therefore the ratio of UFA + BFA to SFA was smaller in the treated cocci than in the 0-time controls. On the other hand, as compared with the 2-h control, $1 \times \text{MIC}$ of methicillin induced a significant increase in the proportion of n-C16:0, ai-C17:0, n-C18:1, ai-C19:0, n-C19:0, and i-C26:0, with a fall in n-C12:3, ai-C15:0, and n-C20:0. There seemed to take place a faster incorporation of the branched fatty acids longer than C16 into the PA of the methicillin-treated cocci and a prevention of the release of n-C18:1, resulting in a higher ratio of UFA + BFA to SFA in the PA of these cells than in the 2-h controls. For this reason, the fluidity of the PA may also be higher in the cocci exposed to $1 \times \text{MIC}$ of methicillin.

Fatty acid distribution of phosphatidylglycerol. The effect of two concentrations of methicillin on the FA composition of the PG in exponentially growing cells of *S. aureus* 5814S is demonstrated in Table II.

As can be seen, the composition of FA in the PG of the untreated cocci hardly changed during the log-phase, since only an increase in n-C12:3 and a decrease in n-C16:1 could be observed. The main FAs were ai-C15:0 and n-C20:0. In the control cells of both ages the PG seemed to be extremely fluid due to the high ratio of UFA + BFA to SFA. When $\frac{1}{2} \times \text{MIC}$ of methicillin was added to the cultures, the proportion of i-C14:0, ai-C15:0, and n-C16:1 decreased substantially, while that of n-C12:3, ai-C17:0, and particularly the proportion of n-C20:0 increased markedly as compared with the 0-time values. Thus, the ratio of UFA + BFA to SFA became significantly less than in the PG of the 0-time control, resulting in a decrease in the fluidity and consequently a tighter packing of the PG. In relation to the 2-h control, there was a fall in n-C12:3 and ai-C15:0, and an increase in ai-C17:0 and n-C20:0. In other words, incorporation of long-chain saturated fatty acids into the PG seemed to increase under the effect of $\frac{1}{2} \times \text{MIC}$ of methicillin.

This tendency became stronger in the presence of $1 \times \text{MIC}$ of methicillin. Compared to the FA distribution of the 0-time control PG, the treated cocci reduced the proportion of i-C14:0, ai-C15:0, and n-C16:1, whereas they increased that of n-C14:0, ai-C17:0, n-C18:0, ai-C19:0, n-C19:0, and n-C20:0.

Table II

Fatty acid composition of phosphatidylglycerol in exponentially growing S. aureus 5814S treated with methicillin

Fatty acid	Per cent fatty acid in PG of cocci in cultures			
	of 0-times control	of 2-h control	exposed to	
			$\frac{1}{2} \times \text{MIC}$	$1 \times \text{MIC}$
			of methicillin for 2 h	
n-C12: 3	—	5.5	2.1	—
n-C12: 0 } n-C13: 0 }	—	—	0.3	0.6
i-C14: 0	2.7	—	—	0.3
n-C14: 0	0.4	1.1	1.3	1.2
ai-C15: 0	66.0	67.0	52.8	50.7
i-C16: 0	1.1	0.6	1.2	0.4
n-C16: 0	1.4	1.3	0.9	1.9
n-C16: 1	2.3	—	—	—
ai-C17: 0	2.8	3.7	7.2	6.8
i-C18: 0	0.2	—	0.2	trace
n-C18: 0	4.3	6.3	4.8	7.5
ai-C19: 0	1.9	2.1	2.9	3.1
n-C19: 0	2.4	1.7	3.4	3.8
i-C20: 0	0.4	—	—	—
n-C20: 0	12.2	10.5	22.9	21.4
ai-C21: 0	—	trace	trace	trace
n-C21: 0	0.5	0.1	—	0.7
i-C22: 0	0.4	0.1	—	0.4
n-C23: 0	—	—	—	0.3
i-C24: 0	1.0	—	—	0.9
Unsaturated	2.3	5.5	2.1	0.0
Branched	76.5	73.5	64.3	62.6
Saturated	21.2	21.0	33.6	37.4
Ratio of UFA + BFA to SFA	3.72	3.76	1.98	1.67

Comparing this with the values of the 2-h control, $1 \times \text{MIC}$ of methicillin brought about a fall in the proportion of n-C12: 3 and ai-C15: 0, while an increase in the proportion of ai-C17: 0, n-C19: 0, n-C20: 0, and in that of all the acids longer than C20. In this way, the ratio of UFA + BFA to SFA became smaller, consequently the fluidity of the PG decreased.

Table III

Effect of methicillin on the fatty acid composition of diphosphatidylglycerol in exponentially growing S. aureus

Fatty acid	Per cent fatty acid in DPG of cells in cultures			
	of 0-time control	of 2-h control	treated with	
			$\frac{1}{2} \times \text{MIC}$	$1 \times \text{MIC}$
			of methicillin for 2 h	
n-C13: 0	—	0.1	—	4.3
i-C14: 0	0.7	1.2	1.0	2.2
n-C14: 0	0.8	1.3	0.4	1.8
ai-C15: 0	29.8	43.6	37.1	32.6
i-C16: 0	1.2	1.4	1.1	0.8
n-C16: 0	5.6	2.3	1.9	6.4
n-C16: 1	4.2	2.8	4.3	3.9
ai-C17: 0	4.3	3.3	4.9	4.1
n-C17: 0	0.7	0.9	0.6	0.6
i-C18: 0	0.5	0.5	0.6	0.5
n-C18: 0	8.8	8.4	7.9	9.6
n-C18: 1	4.9	2.2	3.7	3.5
ai-C19: 0	trace	1.6	2.2	2.0
n-C19: 0	4.9	3.1	3.2	3.5
i-C20: 0	0.9	—	trace	—
n-C20: 0	26.4	23.9	26.5	18.4
n-C20: 1	0.7	1.1	1.7	—
n-C21: 0	2.0	1.2	2.0	2.2
i-C22: 0	0.2	—	—	0.3
n-C22: 0	1.1	0.6	0.7	0.9
i-C24: 0	2.0	0.5	—	2.4
n-C24: 0	0.3	—	0.2	—
Unsaturated	9.8	6.1	9.7	7.4
Branched	39.6	52.1	46.9	44.9
Saturated	50.6	41.8	43.4	47.7
Ratio of UFA + BFA to SFA	0.98	1.39	1.30	1.10

Fatty acid distribution of cardiolipin. The effect of two concentrations of methicillin on the FA composition of the DPG in the cells of *S. aureus* 5814S growing logarithmically is exhibited in Table III.

The FA distribution in the DPG of the control cocci changed during the 2-h period of the exponential phase. The proportion of ai-C15: 0 increased at

the expense of the long-chain fatty acids. Consequently, the ratio of UFA + BFA to SFA increased and the DPG became more fluid.

Administering $\frac{1}{2} \times \text{MIC}$ of methicillin to the cultures caused an increase in the proportion of ai-C15:0, and ai-C19:0, while a fall in n-C16:0 relative to the 0-time control values. The ratio of UFA + BFA to SFA became somewhat higher. In comparison with the 2-h control, the proportion of ai-C15:0 was slightly reduced and, at the same time, that of n-C16:1, n-C18:1, and n-C20:0 was slightly elevated. On the whole, the changes induced by $\frac{1}{2} \times \text{MIC}$ of methicillin did not alter significantly the ratio of UFA + BFA to SFA. In relation to the 0-time control, when $1 \times \text{MIC}$ of methicillin was added to the cultures, the proportion of n-C13:0, i-C14:0, n-C14:0, ai-C15:0, and ai-C19:0 was moderately increased, whereas that of n-C20:0 was decreased. Still the changes failed to alter considerably the ratio of UFA + BFA to SFA. On the contrary, as compared to the 2-h control values, the proportion of n-C13:0, i-C14:0, n-C16:0, n-C21:0, and i-C24:0 increased, while that of ai-C15:0 and n-C20:0 decreased. In this way, the ratio of UFA + BFA to SFA was smaller in the presence of methicillin than in the controls of corresponding age. Nevertheless, the action of methicillin proved to be the weakest on the FA distribution of the DPG among the acid phospholipids.

Fatty acid distribution of lysylphosphatidylglycerol. The effect of two concentrations of methicillin on the FA distribution of LPG in exponentially growing cells of *S. aureus* 5814S is displayed in Table IV.

The predominant fatty acids in the LPG of the 0-time cultures were ai-C15:0, n-C18:0, and n-C20:0. In the LPG of the untreated cocci the proportion of n-C12:3, n-C16:0, and ai-C17:0 rose, while that of ai-C15:0, n-C18:0, and all the fatty acids longer than C18 fell during the log-phase. In the 2-h control the main FAs in LPG were the ai-C15:0, n-C16:0, ai-C17:0 and n-C20:0. Thus, all changes resulted in an increase in the ratio of UFA + BFA to SFA so that the fluidity of LPG was increased.

When $\frac{1}{2} \times \text{MIC}$ of methicillin was added to the cultures, the proportion of ai-C15:0 was unchanged, while that of n-C12:3, n-C16:1, ai-C17:0 were slightly, and n-C20:0 considerably, increased. On the other hand, the proportion of all the other fatty acids fell. Comparing with the 2-h control values, $\frac{1}{2} \times \text{MIC}$ of methicillin caused an increase in ai-C15:0, n-C18:0, and n-C20:0, and a decrease in n-C14:1, n-C16:0 and ai-C17:0. Thus, more saturated than branched FA were incorporated into LPG so that the ratio of UFA + BFA to SFA became smaller than in untreated cocci. This might be accompanied with some reduction in the fluidity.

Administering $1 \times \text{MIC}$ of methicillin to the cultures resulted in a slight increase in ai-C15:0, ai-C19:0, and n-C20:0, with a marked increase in ai-C17:0, and a slight decrease in the proportion of the other fatty acids. In relation to the 2-h control, there was an increase in ai-C15:0, n-C18:0,

Table IV

Effect of methicillin on the fatty acid distribution in lysylphosphatidylglycerol of exponentially growing S. aureus 5814S

Fatty acid	Per cent fatty acid in LPG of cocci in cultures			
	of 0-time control	of 2-h control	exposed to	
			$\frac{1}{2} \times \text{MIC}$	$1 \times \text{MIC}$
			of methicillin for 2 h	
n-C12: 3	—	2.0	2.0	—
n-C14: 0	2.1	1.9	1.0	—
n-C14: 1	1.0	1.5	—	—
ai-C15: 0	35.8	24.7	36.3	38.3
n-C16: 0	4.9	17.6	3.0	2.4
n-C16: 1	—	—	2.2	—
ai-C17: 0	6.4	25.1	9.2	11.3
n-C18: 0	11.1	6.6	9.5	8.3
ai-C19: 0	4.0	2.7	3.0	4.8
n-C19: 0	4.9	2.3	2.7	6.0
n-C20: 0	20.4	15.6	31.1	24.5
i-C20: 0	1.8	—	—	—
n-C22: 0	3.0	—	—	2.0
n-C24: 0	4.6	—	—	2.1
Unsaturated	1.0	3.5	4.2	0.0
Branched	48.0	52.5	48.5	54.7
Saturated	51.0	44.0	47.3	45.3
Ratio of UFA + BFA to SFA	0.96	1.27	1.11	1.21

ai-C19: 0, n-C19: 0, n-C20: 0, n-C22: 0, and n-C24: 0, while the proportion of the other acids decreased. The incorporation of normal short chain fatty acids into LPG appeared to be slower than that of anteiso acids, approaching the ratio of BFA to SFA to the 2-h control ratio. Therefore, the LPG became somewhat more fluid than in the presence of $\frac{1}{2} \times \text{MIC}$ of methicillin.

Fatty acid distribution of C_{55} -isoprenylphosphate. The effect of two concentrations of methicillin on the FA composition of C_{55} -IP in the cells of *S. aureus* 5814S growing exponentially is shown in Table V. These data are preliminary.

The predominant FA in the C_{55} -IP of the 0-time control were ai-C15: 0, n-C16: 0, n-C18: 0, n-C18: 1, and n-C20: 0. During a 2-h period of the log-phase there was an increase in the proportion of n-C12: 3, ai-C15: 0, and i-C22: 0, so that these FA became the main components, and at the same time

Table V

Fatty acid composition of C₅₅-isoprenylphosphate in exponentially growing S. aureus 5814S treated with methicillin

Fatty acid	Per cent fatty acid in C ₅₅ -IP of cells in cultures			
	of 0-time control	of 2-h control	treated with	
			$\frac{1}{2} \times \text{MIC}$	$1 \times \text{MIC}$
			of methicillin for 2 h	
n-C12: 3	—	20.1	66.4	—
ai-C15: 0	18.3	34.3	23.5	15.7
n-C16: 0	25.8	4.5	2.6	21.4
ai-C17: 0	—	0.5	—	—
n-C18: 0	10.7	2.9	1.6	8.5
n-C18: 1	18.1	1.6	—	20.8
ai-C19: 0	0.4	—	—	0.3
n-C19: 0	6.5	—	—	5.6
n-C20: 0	12.4	6.7	3.0	14.5
ai-C21: 0	—	—	0.4	—
n-C21: 0	trace	1.3	1.0	trace
i-C22: 0	4.2	28.1	1.5	6.9
i-C26: 0	3.6	—	—	6.3
Unsaturated	18.1	21.7	66.4	20.8
Branched	26.5	62.9	25.4	29.2
Saturated	55.4	15.4	8.2	50.0
Ratio of UFA + BFA to SFA	0.81	5.49	11.2	1.0

there was a decrease in the proportion of n-C16: 0, n-C18: 0, n-C18: 1, and n-C20: 0. The ratio of UFA + BFA to SFA thus grew from 0.81 to 5.49, consequently, the C₅₅-IP became extremely fluid.

In the presence of $\frac{1}{2} \times \text{MIC}$ of methicillin, the incorporation of n-C12: 3 into C₅₅-IP was faster and that of ai-C15: 0 was slower than in the absence of the antibiotic. Furthermore, $\frac{1}{2} \times \text{MIC}$ of methicillin prevented the incorporation of i-C22: 0 into C₅₅-IP. All the changes resulted in a significant increase in the ratio of UFA + BFA to SFA, leading to a 2-fold increase in the fluidity of the C₅₅-IP.

Adding $1 \times \text{MIC}$ of methicillin to the cultures brought about an almost complete fixation of the FA composition in C₅₅-IP at the 0-time distribution level. The ratio of UFA + BFA to SFA was only 1.0 corresponding to a low fluidity.

Discussion

The first report on the effect of methicillin on the content and FA composition of phospholipids in *S. aureus* came from our laboratory in 1971 [33]. At the time the experiments indicated that 0.25 $\mu\text{g/ml}$ of methicillin altered the phospholipid distribution in *S. aureus* 5814S during a 18-h treatment. On the other hand, in the presence of 1 $\mu\text{g/ml}$ of methicillin the FA composition of the main phospholipids in *S. aureus* 9997P-S differed from that in the cells of the control cultures. Later HEBELER *et al.* [17] demonstrated an enhanced incorporation of lysine into LPG under the effect of penicillin-G.

On the basis of the results we have supposed that methicillin and the similar antibiotics act simultaneously on the peptidoglycan and phospholipid synthesis in sensitive *S. aureus*. Furthermore, death of the cocci in the presence of methicillin has been attributed to the combination of the two effects [20, 26, 33, 34].

The role of changing lipids in the killing action of methicillin has previously been discussed from the point of view of membrane composition and permeability [35]. In the present paper the significance of the changes in the amount and FA composition of phospholipids in *S. aureus* under the influence of the two concentrations of methicillin were studied from the point of view of the interactions of lipids with methicillin and with the cell wall synthesizing and hydrolyzing enzymes.

Exposing the cocci of the exponentially growing cultures of *S. aureus* to $1/2 \times \text{MIC}$ of methicillin the majority had a possibility to survive and to multiply [20, 26, 34]. During a 2-h treatment these cocci increased significantly their PL content. According to BARSUKOV *et al.* [36] in pronase treated protoplasts of *Micrococcus lysodeikticus* the DPG is distributed almost evenly between the inner and outer surfaces of the protoplasmic membrane whereas the PG is located predominantly on the outer surface and the phosphatidyl-inositol on the inner one.

Considering the situation in *S. aureus*, the outer surface charge of the membrane may be determined by the molar ratio of the acid phospholipids to the alkaline ones. This ratio ranges between 3.78–3.95 in the presence of $1/2 \times \text{MIC}$ of methicillin and does not differ significantly from that in the control cells so that the membrane has a net negative charge at cultivation (pH 7.0–7.2) [26]. Thus, on the one hand, the 3-carboxyl group of methicillin is only partially ionized on the surface of the membrane, and on the other hand, the significantly thicker and more packed lipid part of the membrane may delay the access of methicillin to its target. This conclusion is in accordance with that of RETSEMA and RAY [37], and is supported indirectly by the findings of PADFIELD and KELLAWAY [38] showing the penicillins to bind

with phospholipids which may influence the further transport of penicillins.

On the contrary, when $1 \times \text{MIC}$ of methicillin is added to the cultures it prevents further growth and the majority of cocci are condemned to death. In this situation a series of opposite events take place. The most important are the significantly reduced amounts of phospholipids in the cells and a shift from a strong negative charge to a weak one of the phospholipid membrane. Consequently, more methicillin can be ionized and its access may be easier to the target.

The interaction of phospholipids with the cell wall synthesizing and hydrolyzing enzymes seems to be of great importance in the mode of action of methicillin. Methicillin and the other beta-lactam antibiotics are known to inhibit the transpeptidation reaction, resulting in an accumulation of wall materials [39, 40] and in a disorder of septum formation and in the separation of the daughters [20, 34]. Furthermore, a number of wall synthesizing enzymes require phospholipids for their activity [20, 41, 42]. Still, the effectiveness of lipids depends on their amount and on the structure and on the fluidity of their hydrocarbon chains [42–44]. In this respect, a variety of SFA, UFA and BFA has been shown to slow down or inhibit the growth of *S. aureus* [45–48]. On the other hand, CLEVELAND *et al.* [49] found the fully acylated lipoteichoic acid of *S. faecalis* to inhibit autolysis of walls of the same organism, while the deacylated lipoteichoic acid failed to inhibit wall autolysis.

In the light of these observations our results may be interpreted as follows. Cell wall synthesizing enzymes in *S. aureus* 5814S seem to require for their normal activity strongly negative-charged membrane lipids with predominance of PG of high fluidity. Moreover, these enzymes appear to prefer the prevalence of ai-C15:0 acid among the FAs of the membrane phospholipids.

When $1/2 \times \text{MIC}$ of methicillin was added to the cultures, although the charge of the phospholipid membrane remained the same as that of the control, and the amount of the phospholipids increased 1.4-fold [20, 26], the physical state of the hydrocarbon portions of these lipids changed from a high fluidity to a low one. This may be accompanied by a decrease in effectiveness of these lipids as activators of the wall synthesizing enzymes, consequently, the activity of these enzymes may be reduced. On the other hand, the big amount of fully acylated phospholipids in which more than 30% of the FAs is longer than C18, may partially inhibit the septum autolysis leading to the retardation of cell division [20, 34].

On the contrary, the addition of $1 \times \text{MIC}$ of methicillin to the cultures resulted in a significant reduction of the amount of the total PL and of the negative charge of the phospholipid membranes and of the fluidity of the hydrocarbon chains of the phospholipids. This conclusion is supported by the findings presented and by the facts that the ratios of PG to LPG were 3.03, 1.81 and 2.34 in the 0, 1st and 2nd hours of treatment, respectively [35], and

the ratios of the total acid phospholipids to the total alkaline ones were 3.43, 2.14 and 3.00, while those in the controls were 3.37, 3.50 and 3.86, respectively [26]. Under these conditions neither the amount of phospholipids nor their fluidity seem to be sufficient for the normal activity of the wall synthesizing enzymes. Consequently, the activity of those enzymes that are not directly inhibited by methicillin including the septum autolysins is slowed down then stops. In addition, the transpeptidases are almost fully inhibited [20, 39, 40]. This conclusion is in accordance with the ultrastructural features of the cocci [20, 34]. On the other hand, in the methicillin-treated cells the inhibitory effect of the low amount of acylated phospholipids on the endogeneous autolysins may not be sufficient to suppress the activity of these enzymes. Thus their activity may be derepressed and this may contribute to the fast lysis of the cocci.

These manifold effects of the changing membrane lipids accelerate the decay of staphylococci in the presence of $1 \times \text{MIC}$ of methicillin.

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PROTICINE TYPING OF SEROLOGICALLY DEFINED *PROTEUS* STRAINS

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Serologically defined *Proteus* strains including 120 *P. vulgaris*, 356 *P. mirabilis*, 47 *P. morganii*, 36 *P. rettgeri* and 66 *P. inconstans* cultures isolated from a wide variety of sources, were classified by proticine (bacteriocine) typing. Using CRADOCK-WATSON's set of indicator and producer strains, the isolates were tested for proticine production (P type) and for proticine sensitivity (S type). *P. vulgaris* was typable by the P method in 4.1%, by the S method in 41.6%. The respective percentages for *P. mirabilis* were 60.1 and 48.9. Most strains of *P. morganii*, *P. rettgeri* and *P. inconstans* were untypable by both methods. A correlation has been demonstrated between P/S types and certain serogroups and serotypes of *P. mirabilis*. It has been concluded that, in combination with the determination of O and H antigens, the P/S method of proticine typing has a high differentiating value in the epidemiological tracing of *P. vulgaris* and *P. mirabilis*.

In addition to serological and phage typing and grouping by the Dienes reaction, the bacteriocine (proticine, proteocine) method has increasingly been used for the epidemiological characterization of *Proteus* isolates. On the basis of bacteriogenicity tested on 24 indicator strains, CRADOCK-WATSON divided the *Proteus* strains into 10 types [1]. COETZE *et al.* [2] showed that bacteriocines of *P. vulgaris* acted on *P. vulgaris* and *P. mirabilis* strains but not on other *Enterobacteriaceae* species. Authors using CRADOCK-WATSON's indicator strains reported considerably varying results. TRACY and THOMPSON [3] demonstrated that bacteriocinogenic cultures acting on the CRADOCK-WATSON indicators occurred only in 6%, whereas indicator strains chosen from their own material allowed the detection of proticinogenic cultures at a higher frequency. In contrast, PITT [4] was able to type 75–80% of *Proteus* isolates by the method of CRADOCK-WATSON.

There were several attempts to improve the CRADOCK-WATSON scheme. Testing sensitivity against the products of 12 strains, AL-JUMAILI [5, 6] typed all *P. vulgaris* and *P. mirabilis* isolates. Whether his results reflected pure proticine action or some of the preparations were contaminated with phages, has been questioned by SENIOR [7].

SENIOR [7] developed a new scheme based on a combined determination of proticine production by, and proticine sensitivity of, the isolates. On the basis of proticine production detected on a set of 14 indicator strains (P type) and proticine sensitivity to 13 proticine-producing reference strains, SENIOR differentiated 90 distinct P/S types. In view of the fact that this system allows

a more delicate discrimination of *Proteus* strains than do other methods, we have chosen the P/S classification to investigate the correlation between serological and proticene types and subdivide the KAUFFMANN-PERCH serotypes into smaller units.

Materials and methods

Strains. Antigenically defined *Proteus* strains maintained in the Hungarian National Collection of Medical Bacteria, National Institute of Hygiene, Budapest, were used (Table I). In addition, 140 *P. mirabilis* strains isolated freshly in the Salgótarján laboratory from miscellaneous clinical samples, were included in the study. Twenty-four indicator strains and 10 proticene producer reference strains of the CRADOCK-WATSON scheme were kindly supplied by Dr. T. L. PITT, Cross Infection Reference Laboratory, Central Public Health Laboratory, Colindale, London.

Culture medium. MacConkey agar was prepared using the following ingredients. Bacto peptone (Difco), 17 g; Proteose peptone No. 3 (Difco), 3 g; lactose, 10 g; taurocholate (Fluka), 3.9 g; NaCl, 5 g; neutral red, 0.03 g; crystal violet, 0.001 g; agar, 20 g; distilled water, 1000 ml. After adjusting the pH to 7.1, the medium was autoclaved at 121 °C for 15 min.

Typing by proticene production (P type). The isolate was cultured in broth for 4 h then streaked diametrically with cotton swab across the surface of MacConkey agar poured in Petri dishes 90 mm in diameter. After incubation at 30 °C for 20 h, the growth was removed with a glass slide and a piece of cotton wool soaked in chloroform was placed in the lid of the Petri dish. After standing for 30 min, the cotton wool was removed and the plates were left to stand open for 1 h. Then two-hour cultures of the indicator strains were pipetted into the wells of Takátsy's microtitrator plates and, using a multiple inoculator supplied with 8 loops, were streaked across the plates at right angles to the growth of the isolate under test.

Typing by proticene sensitivity (S type). Using the same technique, the standard producer strains were inoculated diametrically onto MacConkey plates. The growth was killed with chloroform and scraped off with a slide. Employing the multiple inoculator, each plate was then seeded with 8 different isolates to be typed.

Readings were performed for both kinds of test after incubation overnight at 37 °C. Inhibition of growth was recorded and P and S types were identified as described in SENIOR's scheme [7].

Table I
Antigenically defined Proteus strains used for proticene typing
(Total number of strains, 625)

Species	Source and reference	No. of strains
<i>P. vulgaris</i> and <i>P. mirabilis</i>	Antigen reference strains of KAUFFMANN and PERCH, Denmark [8]	75
	Antigen reference strains of LÁNYI, Hungary [9, 10]	35*
	Strains collected by LÁNYI, Hungary [9, 10]	366**
<i>P. morgani</i>	Antigen reference strains of the RAUSS-VÖRÖS schema [11-14], Hungary	47
<i>P. rettgeri</i>	Antigen reference strains of NAMIOKA and SAKAZAKI, Japan [15]	36
<i>P. inconstans</i>	Antigen reference strains of EWING <i>et al.</i> , USA [16]	66

* Source of strains: infants' faeces, 12; healthy adults' faeces, 10; faeces of children and adults with enteritis, 1; non-faecal human samples, 12

** Source of strains: infants' faeces, 199; healthy adults' faeces, 65; faeces of children and adults with enteritis, 23; non-faecal human samples, 88

Results

Incidence of strains typable by proticine production and sensitivity. Table II shows the number of strains belonging to different *Proteus* species typable by proticine production (P⁺), by proticine sensitivity (S⁺) or by both features. *P. vulgaris* strains unfrequently (4.1%) produced proticines acting on the indicator strains, but were in 41.6% sensitive in testing with the standard producer cultures. *P. mirabilis* isolates were typable by the P method in 60.1% (214 out of 356) and in 48.9% by the S method (174 out of 356).

As seen in Table II, most strains of other *Proteus* species were untypable. *P. rettgeri* and *P. inconstans* failed to produce proticines active on the indicator strains and the 2 proticinogenic *P. morganii* strains exhibited activity spectra different from those of the reference strains.

Correlation between serotype and proticine type. Table III shows the proticine-type distribution of *P. vulgaris* and *P. mirabilis* strains defined by O and H antigens. Of P-type cultures, P5 was most frequently met with (98 strains). Types P2 (26 strains), P6 (21 strains), P3 (17 strains), P7 (10 strains) and P4 (8 strains) were next in order. Types P9 and P10 were not encountered. The number of unclassifiable proticine-producer cultures (uc) was 21. All but five proticine-producer isolates belonged to *P. mirabilis*. The P types of the five *P. vulgaris* strains were P5 (serotype 15: 1), P6 (serotype 1: — and 2: 1) and Puc (serotypes 1: 1 and 25: ND).

According to sensitivity typing, most isolates fell into type S5 (83 strains) followed by types S1 (39 strains), S4 (47 strains), S7 (36 strains), S10 (27 strains), S2 (25 strains), S3 (21 strains), S8 (12 strains) and S6 (5 strains). Nearly half of the 50 *P. vulgaris* typable by proticine sensitivity was inhibited by two or more of the standard producer strains.

As shown in Table III, there is some correlation between serotype and P/S type. For example, serotype 3: — and 3: 1 strains belonged mostly to P5/S0,

Table II

Incidence of Proteus strains typable on the basis of proticine production (P) and proticine sensitivity (S)

Species	Total number of strains	Typable strains					Untypable strains	
		P+/S-	P+/S+	P-/S+	total		P-/S-	
					No.	per cent	No.	per cent
<i>P. vulgaris</i>	120	2	3	47	52	43.3	68	56.7
<i>P. mirabilis</i>	356	112	102	72	286	80.3	70	19.7
<i>P. morganii</i>	47	2	—	4	6	.	41	.
<i>P. rettgeri</i>	36	—	—	1	1	.	35	.
<i>P. inconstans</i>	66	—	—	10	10	.	56	.

Table III

Distribution of P. vulgaris and P. mirabilis strains according to serotypes and P/S types
(Total number of strains, 476)

Serotypes	Species	No. of strains	P/S type (parenthesized figures: no. of strains)
1: —	1	3	6/2, 3 (1), 0/0 (2)
1: 1	1	7	uc/0 (1), 0/5 (1), 0/0 (5)
1: 6	1	1	0/5 (1)
2: 1	1	2	6/0 (1), 0/0 (1)
3: —	2	10	5/0 (29), 0/0 (1)
3: 1	2	53	5/0 (36), 5/2 (2), 5/4 (5), 5/7 (2), 5/8 (1), uc/0 (2), 0/4 (1), 0/5 (1), 0/0 (8)
3: 2	2	13	2/7 (5), 2/0 (2), 2/5 (1), 1/0 (1), 0/4, 10 (1), 0/7 (2), 0/5 (1)
3: 6	1	2	0/2, 5 (2)
3: ND	2	1	0/0 (1)
4: 1	1	3	0/0 (3)
4: 8	1	1	0/0 (1)
4: 16	1	1	0/0 (1)
5: 1	2	5	6/2, 3 (3), 1/2, 3 (2)
5: 2	2	1	2/1, 5 (1)
6: 1	2	1	0/0 (1)
6: 3	2	21	6/4, 10 (9), 6/4, 5 (1), 2/4, 10 (2), 5/10 (1), 5/0 (1), uc/2 (1), 0/4, 10 (2), 0/7 (1), 0/0 (3)
6: 4	2	1	0/7, 10 (1)
7: 1	2	1	0/0 (1)
7: 3	2	1	2/8 (1)
7: 6	2	1	0/7 (1)
8: 1	1	1	0/0 (1)
9: 1	2	3	4/10 (1), 6/4 (1), 0/5 (1)
9: 2	2	4	1/2, 10 (1), uc/3 (1), uc/7, 10 (1), 0/5 (1)
9: ND	2	1	4/10 (1)
10: 1	2	14	7/1 (4), 7/5 (3), 7/1, 5 (1), 2/7 (1), 0/1 (2), 0/5 (1), 0/1, 5, 6 (1), 0/0 (1)
10: 2	2	5	3/5 (2), 3/0 (1), 0/1, 3, 4, 8 (1), 0/0 (1)
10: 3	2	5	0/1 (2), 0/1, 5 (1), 0/5 (2)
10: 4	2	3	0/0 (3)
10: 5	2	2	0/1 (1), 0/2 (1)
11: 2	2	2	1/0 (1), 0/0 (1)
11: 3	2	1	0/5 (1)
11: 6	2	1	0/0 (1)
12: 1	1	4	0/7 (1), 0/0 (3)
12: 8	1	2	0/4 (1), 0/0 (1)
12: ND	1	3	0/4 (1), 0/4, 5 (2)
13: 1	2	17	0/5 (11), 3/5 (1), 2/5 (1), uc/5 (1), 0/5, 10 (1), 0/2, 3 (1), 0/0 (1)
13: 2	2	5	5/4 (3), 5/0 (2)
13: 3	2	3	6/5 (2), 6/3 (1)
13: 4	2	1	3/0 (1)
13: 5	1	1	0/4, 8 (1)
13, 30: 1	2	4	6/5 (1), 0/5 (2), 0/7 (1)
13: ND	1	1	0/0 (1)
	2	3	0/7 (1), 0/0 (2)
14: 1	2	1	0/0 (1)
14: 3	2	1	uc/0 (1)
14: ND	1	1	0/0 (1)
15: 1	1	1	5/1, 4 (1)
15: 3	2	3	uc/7 (2), 0/7 (1)
15: 7	1	1	0/2 (1)

Table III (continued)

Serotypes	Species	No. of strains	P/S type (parenthesized figures: no. of strains)
16: 1	1	1	0/4,5 (1)
16: 6	1	1	uc/7 (1)
16: 14	2	1	0/0 (1)
16: ND	1	1	0/0 (1)
17: 1	2	1	uc/0 (1)
17: 10	1	1	0/0 (1)
18: 1	2	1	5/0 (1)
19: 1	1	5	0/3 (1), 0/5 (1), 0/7 (1), 0/0 (2)
	2	1	0/0 (1)
19: 3	2	1	3/0 (1)
19: 5	1	1	0/8 (1)
19: 6	1	1	0/0 (1)
19: 11	1	1	0/0 (1)
19: 12	1	1	0/1,5 (1)
19: ND	1	1	0/3,5 (1)
20: 1	2	1	0/0 (1)
20: 2	2	1	4/0 (1)
21: 1	1	7	0/5 (2), 0/4 (1), 0/7 (1), 0/0 (3)
	2	1	0/7 (1)
22: 1	1	1	0/0 (1)
22: ND	1	1	0/0 (1)
23: 1	1	3	0/1 (1), 0/1,5 (1), 0/1,8 (1)
	2	1	0/1 (1)
23: 2	1	1	0/1,5 (1)
	2	1	3/0 (1)
23: 3	2	1	0/0 (1)
23: 12	1	1	0/1,5 (1)
23: ND	1	2	0/5 (1), 0/0 (1)
	2	2	3/8 (2)
24: 1	2	1	uc/5 (1)
24: 3	2	2	1/0 (2)
24: 4	2	1	3/0 (1)
24: 13	2	3	0/4 (1), 0/0 (2)
25: 1	1	1	0/0 (1)
25: ND	1	1	uc/2,3,5 (1)
26: —	2	2	0/5 (1), 0/0 (1)
26: 3	2	29	2/1 (8), 2/0 (3), uc/0 (1), 0/1 (3), 0/4 (2), 0/0 (12)
26: 6	1	3	0/3 (1), 0/0 (2)
26: ND	1	3	0/0 (3)
	2	1	2/1 (1)
27: 1	2	2	0/0 (2)
27: 2	2	5	1/0 (2), uc/0 (1), 0/0 (2)
27: ND	1	1	0/0 (1)
28: 2	2	14	5/7 (3), 5/6 (3), 5/1,2 (1), 4/7 (1), uc/5 (2), uc/7 (1), 0/0 (3)
28: 3	2	5	0/8 (2), 3/8 (1), 0/0 (2)
28: 6	2	2	8/0 (2)
28: ND	2	2	5/0 (1), 7/0 (1)
29: 13	2	5	uc/0 (1), 0/1,2 (1), 0/4 (1), 0/5 (1), 0/0 (1)
30: 1	2	5	4/2,3,5 (1), 0/2,3 (1), 0/4 (1), 0/6 (1), 0/0 (1)
30: 2	2	2	1/5 (1), 0/0 (1)
30: 13	2	1	0/0 (1)
30: 15	2	1	5/0 (1)
31: 1	1	2	0/2 (1), 0/0 (1)
31: 2	2	6	5/0 (2), 5/7 (2), uc/0 (1), 0/7 (1)
31: ND	2	1	5/0 (1)
32: 1	1	3	0/4,7 (1), 0/0 (2)

(contd.)

Table III (continued)

Serotypes	Species	No. of strains	P/S type (parenthesized figures: no. of strains)
32: 3	2	1	7/3,8 (1)
32: 6	1	1	0/0 (1)
33: 1	2	2	4/10 (1), 4/7 (1)
33: 2	2	6	3/0 (3), 3/2,3,5 (1), 0/0 (2)
33: 3	2	1	0/0 (1)
33: 4	2	1	0/0 (1)
33: ND	2	1	0/7 (1)
34: 1	2	1	0/0 (1)
34: 6	1	2	0/0 (2)
34: 8	1	1	0/0 (1)
35: 2	2	1	4/0 (1)
36: 3	2	1	0/10 (1)
36: 6	1	2	0/0 (2)
37: 17	1	1	0/5 (1)
38: 1	2	1	uc/0 (1)
38: 2	2	1	uc/5 (1)
38: 6	2	1	0/0 (1)
39: 18	1	1	0/1 (1)
40: 2	2	1	1/0 (1)
40: 4	2	1	0/1,3 (1)
41: 1	2	1	5/0 (1)
41: 4	2	1	3/0 (1)
41: 17	1	1	0/0 (1)
42: 1	1	3	0/0 (3)
42: 2	2	1	0/5 (1)
43: 2	2	1	0/2,3,5 (1)
43: ND	1	1	0/3,10 (1)
44: 19	1	1	0/4 (1)
45: 11	1	2	0/0 (2)
45: ND	1	1	0/0 (1)
	2	1	0/4,10 (1)
46: 17	1	1	0/5 (1)
47: 1	1	2	0/5 (2)
48: 1	2	1	0/0 (1)
49: 2	2	1	0/7 (1)
ND*	1	21	0/4,5 (5), 0/5,10 (1), 0/10 (1), 0/2,5 (1), 0/5 (1), 0/0 (12)
ND**	2	13	1/2 (1), 3/0 (1), 6/0 (1), uc/5 (1), uc/7 (1), 0/4,10 (1), 0/5(2), 0/0 (5)

* ND: 1 (3), ND: 5 (2), ND: 6 (5), ND: 8 (2), ND: ND (9)

** ND: 0 (1), ND: 2 (4), ND: 3 (2), ND: 5 (2), ND: 6 (1), ND: 13 (1), ND: ND (2)

Serotype. Figures ahead of colon mean the O antigen, those after the colon, the H antigen

ND = antigen different from those described by KAUFFMANN and PERCH [8]

— = H antigen absent

Species. 1 = *P. vulgaris* (120 strains); 2 = *P. mirabilis* (356 strains)

P/S type. 0 = P type: strains not producing proticines detectable on the standard indicator strains; S type: strains not sensitive to proticines of the standard producer strains

uc = strain producing unclassified proticine

whereas serotype 3: 2 cultures produced frequently P2 proticine. The correlation was independent of the origin of the cultures, as many strains belonging to the same serotype and proticine type had been isolated from a variety of sources in Hungary. Moreover, the corresponding serotypes isolated by KAUFFMANN and PERCH in Denmark showed identical proticine spectra, e.g.

serotype 3a,b: 1a,c,e belonged to type P5/S0, 3a,b: 2a,b,e,f to P2/S0. In serotype 6: 3 proticine type P6/S4,10 predominated. Serotype 10: 1 strains frequently belonged to P7, whereas P3 was characteristic of 10: 2 strains. Serotype 13: 1 strains fell in their majority to P0: S5, 13: 2 to P5 and 13: 3 to P6. Proticine producer isolates of P2 were frequently encountered in antigenic group O26 and those of type P5, in O28.

In addition to strains isolated 20–30 years ago (Table I), 140 *P. mirabilis* strains cultured recently from miscellaneous clinical material were tested. Most of these strains belonged to serogroups O3, O10, O11, O13, O13,30, O23, O28, O30 and O31. They fell partly to P/S types characteristic of the corresponding O group (e.g. P2/S0 in serogroup O3, P7/S1 in serogroup O10, etc.), and partly represented many P/S types not encountered in the KAUFFMANN-PERCH and in the LÁNYI collection. Fifty of these isolates contained O antigens not determinable according to the KAUFFMANN-PERCH scheme; they belonged to 18 different P/S types.

Discussion

Data in the literature and the present findings agree in that on the basis of proticine production against CRADOCK-WATSON's indicator strains (P method) mainly *P. mirabilis* strains can be typed. Other methods [17] and mitomycin C induction [5] also revealed that members of this species produced proticines the most frequently.

Typing by the sensitivity of the isolates to standard proticines (S method) was also the most effective for *P. mirabilis*, and *P. vulgaris*, too, was fairly frequently typable by this method.

For the remaining species of the genus, neither the P nor the S method is of value. Recently, *P. rettgeri* has been typed with its own selected indicator strains [18] and *P. inconstans* (*Providencia*) strains have been classified by their provicine sensitivity [19].

Proticine production is influenced by several factors, such as ingredients of the medium, conditions of incubation [20] and the effect of the antismearing agent in the medium [21]. SENIOR [22] has shown that certain proticine types are associated with urinary infection.

Strains examined in our study were mostly of faecal origin. The frequent incidence of certain P/S types (e.g. 5/0, 6/4,10, 0/5, 2/1) in our material is explained by the fact that *P. mirabilis* serogroups O3, O6, O10, O13 and O26, correlated with these P/S types, predominated in hospitalized patients [10, 11] and were thus represented in the collection by more strains than the other serogroups. There was, however, no significant difference in the P/S type of strains isolated from healthy persons and from patients with enteritis. A considerable amount of our strains was untypable by the P, S

or both methods. Part of these cultures may belong to the three new types described by SENIOR [7].

A large number of serotypes was associated with a single P/S type (Table III), because these serotypes were represented only by one strain or by a few strains mostly of common origin. As to the correlation between proticine type and antigenic structure, strains showing the same features but originating from different sources have been considered. Correlations presented in the Results existed not only with the O antigen, but also with the H antigen. This finding differs from that described for *Pseudomonas aeruginosa*, in which there appeared to be a correlation between pyocine type and O antigen but not between pyocine type and H antigen [23].

Our results have confirmed the differentiating value of the P/S method of proticine typing. Combined with serological typing, the method offers a most delicate means for epidemiological marking: by the use of the simplified KAUFFMANN-PERCH schema and the CRADOCK-WATSON set of proticine reference strains, in the present study *P. vulgaris* and *P. mirabilis* isolates were divided into not less than 261 units.

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INCIDENCE IN ENTERITIS OF *ENTEROBACTERIACEAE* ISOLATES POSSESSING HUMAN COLONIZATION FACTOR ANTIGEN. NEW HUMAN COLONIZATION FACTORS

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Of 462 *Enterobacteriaceae* strains including 435 *Escherichia coli* isolated from 250 patients, 298 haemagglutinating (HA) cultures were classified into 36 different HA groups. Sixteen of them belonged to Evans's I or II groups, although none possessed CF I or CF II antigen detectable by slide agglutination. Seventy-seven strains showed 4+ mannose resistant (MR) HA with human (53), bovine (2), chicken (6), guinea pig (7) or human and guinea pig (9) erythrocytes. These strains were significantly more frequent in patients under one year of age. Eighty-eight percent of the typable strains belonged to *E. coli* serogroups O1, O2, O4, O6, O18. HA positivity and fimbrial structures were correlated in 2 isolates (15/1 O18a, c: K77: H-; 12/2/1 O1: K1: H+). Fimbriae of the two strains exhibited adhesive properties. Their fimbrial antigens differed serologically from each other and from those of the reference strains H 10407 and PB 176. Forty-nine of 4+ human MRHA strains showed variable reactions in the two sera for the new fimbrial antigens.

The pathogenicity of enterotoxigenic *Escherichia coli* is proved by demonstrating its enterotoxicity and/or colonization factor(s). Detection of enterotoxigenicity is a complicated and time consuming procedure. The haemagglutination (HA) typing system established by EVANS *et al.* [1] is a simple method for the screening of strains possessing human colonization factor antigens (CFA I, CFA II).

Our aim was (i) to show the incidence of CFA I and CFA II positive enterotoxigenic strains in Hungary; (ii) to define the application of the method for proving pathogenicity by means of HA typing.

In the course of investigations, 77 out of 462 of our strains exhibited 4+ mannose resistant (MR) HA occurred mainly with human erythrocytes.

Materials and methods

Strains examined were isolated in our laboratory between April 1979–June 1979 from faecal samples of 250 persons. Out of the 462 *Enterobacteriaceae* strains, 435 belonged to *E. coli*, the rest to *Salmonella* (9), *Shigella* (7), *Klebsiella* (6), *Proteus* (3) and *Citrobacter* (2). Further strains tested were: *E. coli* H 10407 (CFA I), H 10407 P (CFA-), *E. coli* PB 176 (CFA II), PB 176 P (CFA-) received from Dr. D. J. EVANS, Jr., 119 (*E. coli* O18a, c: K-; H-, fimbria positive), 119/1 (fimbria negative) received from Dr. B. KUCH [2]. The strains were cultivated on colonization factor antigen (CFA) media [3]. The only modification was that our CFA contained 0.9% agar (Oxoid No. 1).

CFA I, CFA II human colonization factors were determined by HA typing [1] and slide

agglutination in H 10407, PB 176 sera. Sera were produced and absorbed in our laboratory according to EVANS *et al.* [4].

Detection of adhesion factor(s) in strains producing mannose resistant haemagglutination with human erythrocytes. Agglutination. Sera were prepared against two of our isolates (15/1 O18a, c: K77: H-; 12/2/1 O1: K1: H-) according to EVANS *et al.* [4]. These sera and serum 119 were absorbed by living and boiled bacteria and used for tube agglutination. *Fimbria* were shown by electron microscopy [4-6]. CFA preparations were placed on grids, air dried for 15 min and stained with 2% phosphotungstic acid. The preparations were examined in the JEOL model JEM 100 C transmission microscope. *Assay for adherence of E. coli* in the infant rabbit model was performed with strains 15/1, 15/1/P (see Table IV), 12/2/1, 12/2/1/P, 119, 119/1 [2]. Three to five-day-old rabbits were infected intragastrically with 2 ml 10^{-1} dilution of broth incubated at 37 °C for 4 h (2.4×10^7 – 5.2×10^7 bacteria/ml). The animals were sacrificed 18 h postadministration and viable cell counts were determined from the upper part of the proximal jejunum about 10 cm in length. Colony forming units (c.f.u.) were expressed for 1 g of intestine.

Results

Among the 250 persons, 227 suffered from gastrointestinal disease (8 of them were positive for *Salmonella*, and 3 for *E. coli* associated with infantile enteritis), 9 had extraintestinal infections, 8 were either *Salmonella* (1) or *Shigella* (7) asymptomatic carriers, 6 were healthy persons in the environment of patients. Their age distribution is shown in Table I.

Table I

Distribution of patients with 4+ MRHA positive strains

Age (year)	No. of patients examined	No. of patients with MRHA positive strains
Under 1	105	48
1- 2	39	7
3- 6	33	7
6-14	5	0
Above 14	68	15
Total	250	77

HA properties of 462 strains were examined and it was found that besides 4+ HA (77 strains, Table II) 1+ or 2+ HA occurred in both the reference strains (H 10407, PB 176) and the fresh isolates (221 strains). The 298 HA positive strains were classified into 36 different HA groups on the basis of mannose resistant and sensitive HA with erythrocytes of 4 different kinds of animals. Sixteen of the above strains belonged to Evans's I or II group [1]. Slide agglutination in specific sera failed, however, to show CF I or CF II surface antigen. 4+ MRHA exhibited by 77 strains produced MRHA with human (53), bovine (2), chicken (6), guinea pig (7) or human and

guinea pig (9) erythrocytes. The incidence of 4+ MRHA positive strains was significantly more frequent in patients under one year than above 14 years of age ($p < 0.001$, Table I). Distribution of 4+ MRHA positive strains was, *E. coli* serogroups O1 (3), O2 (3), O3 (1), O4 (16), O6 (6), O8 (1), O10 (1), O18 (9), O78 (2), not typable (26), not determined (6), *P. morganii* (2), *Citrobacter* (1).

In subsequent experiments we analysed (i) the properties of 4+ MRHA positive strains; (ii) whether HA properties were connected with the fimbriae of the strains; (iii) adhesive properties of the fimbria; (iv) whether the fimbrial antigens of the different strains were identical in structure.

(i) During 6 months maintenance on Dorset-egg medium there were essential changes in the HA property of the strains (Table II). Strains producing HA with chicken (3) and guinea pig (5) erythrocytes lost their agglutinability. Strains HA positive with both human and guinea pig erythrocytes agglutinated only with human erythrocytes (9).

Table II

HA properties of the same strains examined on April, 1979, and October, 1979

Erythrocytes	No. of 4+ MRHA positive strains	
	April, 1979	October, 1979
Human	53	49
Bovine	2	2
Chicken	6	3
Guinea pig	7	2
Guinea pig + human	9*	—

* All these strains produced HA only with human erythrocytes in October, 1979

Table III

Distribution of human 4+ MRHA positive strains according to their haemolysin production and origin

Haemolysin	Cholera-like disease	Dysentery-like disease	Enteritis	Extraintestinal disease	Total
Positive	1	5	8	8	22
Negative	3	5	17	2	27
Total	4	10	25	10	49

In view of the low number of HA strains positive with bovine, chicken, guinea pig erythrocytes, in further examinations only the connection between haemolysin production of 49 human HA positive strains and the clinical diagnosis was dealt with (Table III).

Haemolysin-producing and non-producing strains occurred in all groups of patients; haemolysin positive cultures predominated only in extraintestinal diseases (sepsis, meningitis).

(ii) The correlation between HA positivity and fimbrial structures of strains 15/1, 12/2/1 and 119 was examined by electron microscopy (Figs 1, 2, 3). All the three strains examined harboured fimbria. The flagellae of strain 12/2/1 could well be distinguished (Fig. 2).

Table IV

Adhesive properties of fimbria positive and negative E. coli strains examined in the infant rabbit model

Designation of strain	No. of		Infective dose	No. of bacteria/g intestine
	experiments	rabbits		
15/1	1	2	3.2×10^7	1.9×10^9 1.6×10^9
15/1/P	2	3	2.4×10^7 2.4×10^7	5.2×10^6 2.7×10^3 1.4×10^4
12/2/1	2	3	5.2×10^7 3.2×10^7	5.6×10^7 1.9×10^4 3.5×10^6
12/2/1/P	2	4	4.4×10^7 4.0×10^7	2.3×10^3 2.3×10^2 4.5×10^1 5.3×10^4
119	3	4	3.6×10^7 2.4×10^7 3.2×10^7	9.3×10^7 4.9×10^7 4.0×10^9 6.5×10^7
119/1	1	2	2.4×10^7	9.6×10^4 4.2×10^3

15/1: *E. coli* O18a,c:K77:H- fimbriated; 15/1/P same, non-fimbriated (passaged and cultured at 22 °C)

12/2/1: *E. coli* O1:K1:H- fimbriated; 12/2/1/P same, non-fimbriated (passaged and cultured at 22 °C)

119: *E. coli* O18a,c:K-:H- fimbriated; 119/1 same, non-fimbriated [2]

(iii) To detect the adhesive property of fimbrial structure, strains 15/1, 15/1/P, 12/2/1, 12/2/1/P, 119, 119/1 were examined in infant rabbits. Colony forming units are shown in Table IV.

(iv) Sera were prepared against strains 15/1 and 12/2/1 and absorbed by homologous, fimbria negative live and heat killed bacteria. Serum 119 was absorbed by 119/1 (see Table IV). Results of homologous and cross agglutinations in absorbed sera are shown in Tables V and VI. Agglutinability of 15/1 and 12/2/1 strains decreased after heating at 65 °C for 60 min, or after several passages and incubation at 22 °C. These properties coincided with the loss of HA positivity of the strains. When incubated at 37 °C, the difference in aggluti-

Table V

Agglutination of different antigen preparations in homologous absorbed sera

Antigen	Sera*		
	15/1	12/2/1	119
CFA 37 °C living	1280	1280	10 240
NA 37 °C living	1280	640	5 120
NA 22 °C living	320	320	640
NA 37 °C 2½ h 100 °C	—	40	—
NA 37 °C 1 h 65 °C	160	40	320

* The sera were absorbed by homologous non-fimbriated living and heated (100 °C, 2½ h) bacteria

CFA = growth from CFA agar

NA = growth from nutrient agar

15/1, 12/2/1, 119: see Table IV

Table VI

Cross agglutination tests for the fimbrial antigens of strains 15/1, 12/2/1, 119, compared to strains H 10 407 and PB 176

Antigen (CFA 37 °C living)	Sera*				
	15/1	12/2/1	119	H 10407	PB 176
15/1	1280	80	640	—	—
12/2/1	—	1280	—	—	—
119	640	80	10 240	—	—
H 10 407	—	80	—	5120	—
PB 176	—	—	—	80	327 600

* Sera were absorbed by homologous living and heated non-fimbriated bacteria

15/1, 12/2/1, 119: see Table IV

H 10407: CFA I positive, fimbriated reference strain

PB 176: CFA II positive, fimbriated reference strain



Fig. 1. Electron micrograph of negatively stained preparation of *E. coli* 15/1. $\times 67\,000$



Fig. 2. Electron micrograph of negatively stained preparation of *E. coli* 12/2/1. $\times 52\,000$

nability was insignificant between the bacteria grown on CFA or those grown on nutrient agar (NA). Strains 15/1, 12/2/1 and 119 failed to cross-react with H 10407 and PB 176. There were some cross reactions between 15/1 and 119.

For analysing the fimbrial antigenic structure of the strains, 15/1 and 119 cross absorptions of previously absorbed fimbria specific sera were performed with fimbriate cultures (Table VII).

Agglutination of the 49 human MRHA positive strains was examined in absorbed fimbrial sera 15/1, 12/2/1 and 119. Results are presented in Table VIII. Eighteen out of 49 strains failed to agglutinate, the remaining 31 showed variable reactions in the three sera. One strain agglutinated in all absorbed sera, but not in physiological saline.

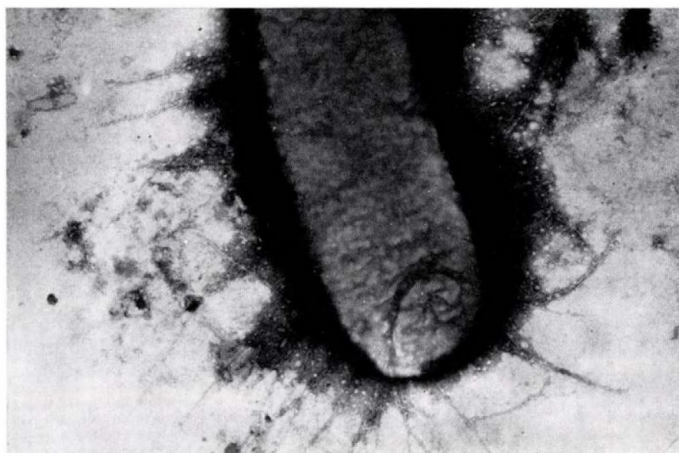


Fig. 3. Electron micrograph of negatively stained preparation of *E. coli* 119. $\times 67\ 600$

Table VII

Antigenic analysis of the fimbriae of strains 15/1 and 119

Antigen (CFA 37°C living)	Sera	
	15/1 absorbed by 15/1/P boiled and living +119 living	119 absorbed by 119/1 boiled and living +15/1 living
15/1	1280	—
119	—	2560

15/1, 15/1/P, 119, 119/1: see Table IV.

Table VIII

Agglutination of MRHA positive strains with human erythrocytes in absorbed antifimbrial sera

No. of strains	Agglutination in absorbed sera*		
	15/1	12/2/1	119
12	+	—	—
18	—	—	+
0	—	+	—
18	—	—	—
1	+	+	+

* Absorption of 15/1 and 119: see Table VII; of 12/2/1: see Table VI
15/1, 12/2/1, 119: see Table IV

Discussion

In the literature different colonization-related antigens have been characterized, e.g. K88, K99 and CFA [1]. Each of these is demonstrable by mannose resistant haemagglutination activity, while type 1 pili produce mannose sensitive haemagglutination [1, 7]. Our experiments were aimed at showing CFA antigen in strains originating from patients with enteritis.

CFA media were adequate for developing the fimbria of strains with colonization factor antigens. Sixteen strains belonged to Evans's group I and II on the basis of HA, but they failed to agglutinate in sera CFA I and CFA II. It is not clear whether this was due to ambiguous results of 1+ and 2+ HA, or to some other reason. In any case, it may be stated that CFA I and CFA II strains were infrequent in Hungary. Screening for strains with CFA I, CFA II antigen, slide agglutination with specific fimbrial immune sera was considered preferable as results with 1+ HA were equivocal and to use erythrocytes of four different animals was laborious.

Two groups of the 77 strains mediating MRHA (one HA positive with human and the other with chicken erythrocytes) corresponded to group IV of Evans's pattern [1]. Strains with type 1 pili also belonged to Evans's group IV but they differed from the former ones in their mannose sensitive HA. Comparing our results with those of DUGUID *et al.* [7] it was noted that our strains probably represented the first or the ninth group of the pattern. Pattern I was the commonest among the strains examined by these authors. As to MRE haemagglutinins, our strains corresponded to the criteria of DUGUID *et al.* [7].

We isolated HA positive strains different from Evans's pattern for bovine and guinea pig erythrocytes. The low number of such strains does not allow to draw further conclusions.

4+ MRHA positive strains occurred significantly more frequently in children under one year of age. There was no relationship between haemolysin production and the fimbrial structures of the strains. The 4+ MRHA positive strains belonged mainly to serogroups O1, O2, O4, O6, O18 (88% of typable strains) which are known to be common in faeces [8]. At the same time only 37 out of 116 MRE+ strains examined by DUGUID *et al.* [7] belonged to these serogroups.

Two representatives of our 4+ MRHA positive strains had heat labile surface antigen. Antigenic analysis of these strains and tube agglutination of 49 4+ MRHA positive strains showed serological differences in their fimbrial antigens. DUGUID *et al.* [7] described similar findings for strains with MRE haemagglutinins.

A relationship between HA property and the presence of fimbria was demonstrated. Adhesive properties of these fimbria could be proved in infant rabbit tests.

While our examinations were in progress, KUCH *et al.* [2] demonstrated MRHA activity with human erythrocytes and presence of fimbriae in their *E. coli* strains isolated from urine. Besides the above results, we have shown that the fimbria of their and our strains adhered to the infant rabbit epithelium.

These strains represented the O serogroups common in faeces. It is known that O groups which frequently occur in urinary infections are also frequently encountered in the faeces of healthy persons [8-12]. Strain 119 isolated by KUCH *et al.* [2] from urine, and strains 15/1 and 12/2/1 isolated in our laboratory from faecal sample of patients with enteritis, all harboured fimbriae. Their adhesive properties represent new colonization factors as serological examinations of their fimbrial antigens revealed that they were antigenically different from each other and differed from the fimbrial antigens of strains H 10407 and PB 176. It may be assumed that these factors stimulated the bacteria to colonize in both the human intestine and the urinary tract. Accordingly, serogroups that they are associated with, are common in the faeces of healthy persons and frequently cause urinary infections.

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IN MEMORIAM



GYÖRGY IVÁNOVICS

Professor György Ivánovics, member of the Hungarian Academy of Sciences, formerly Director of the Institute of Microbiology, University Medical School, Szeged, died on September 1, 1980. He was born in Budapest on June 11, 1904. Educated at Budapest, he entered the Faculty of Medicine in 1922 and graduated in 1928.

His interest in bacteriology began while he was studying medicine and working externally at the Department of Hygiene. After graduation he obtained some training in pathology at St. Rókus Hospital at Budapest. In 1929 he started work at the University of Szeged, employed as an assistant at the Department of Hygiene. He became associate professor in 1935. Between September 1934 and August 1935 he spent a year as a Rockefeller Research Fellow at the Department of Virology and Immunology of Johns Hopkins School of Hygiene, Baltimore, USA. On returning to Hungary he began work at the Institute of Bacteriology and General Pathology where he was appointed professor and chairman in 1940. He retired from that Institute (now named Institute of Microbiology) in 1974.

In 1946 he was elected to correspondent membership of the Hungarian Academy of Science and reelected as an ordinary member in 1956.

The Kossuth Prize (Hungarian National Prize for distinguished work) was awarded to him in 1948 and 1952. He was also a recipient of the Semmelweis and Jancsó Medals. Until 1974 he was the president of the Hungarian Society of Microbiology. He acted as chief editor of *Acta Microbiologica Academiae Scientiarum Hungaricae* and as a member of the editorial board of *Acta Virologica* (Bratislava, Czechoslovakia) and of *Zeitschrift für Allgemeine Mikrobiologie* (Berlin, GDR). He received an Honorary doctorate from Glasgow University in 1972.

During nearly 50 years of research he was interested in different fields of microbiology. He published 204 papers, one monograph and one text-book in virology. He edited text-books in microbiology for medical students in Hungary. Professor Ivánovics was an extraordinary scientist in whom creative spirit was associated with outstanding knowledge. As a junior research worker in 1936 at Johns Hopkins School of Hygiene he developed a tissue culture method for estimating virus infectivity. He organized virus research at his Institute and introduced different tissue culture methods about 1950. He made the fundamental observation with professor Győző Bruckner that the capsule of *Bacillus anthracis* consists of a polymer of D-glutamic acid. One of his important results was the discovery of the megacin which is liberated from some strains of *Bacillus megaterium* after ultraviolet irradiation. Recently he dealt mainly with studies on the mechanism of megacinogeny. His scientific achievements attracted many young investigators for whom he was a constant source of inspiration.

We shall always remember him with honour and devotion.

ILONA BÉLÁDI

INDEX

Körmendy, B., Bánki, M., Szabó, I.: Isolation of Mycobacteria from Contaminated Material on Selective Media	1
Horváth, I., Duncan, W. P., Bullard, J. C.: Cultivation of Pathogenic <i>Treponema pallidum</i> <i>in vitro</i>	7
Ezepchuk, Y. V., Morgan, S. D., Major, P.: A Simple Procedure for Isolation and Purification of A-Type Staphylococcus Enterotoxin	25
Gupta, J. K., Shirkot, C. K., Dhawan, S.: Isolation and Mutation of Cellulolytic Fungi	31
Rudnai, O., Csórián, E., Lányi, B., Ádám, M. M., Milch, H.: Salmonella and Shigella Surveillance in Hungary 1972-1976. I. Salmonella Surveillance	37
Rudnai, O., Straub, I., László, V. G., Hajnal, A., Lányi, B.: Salmonella and Shigella Surveillance in Hungary 1972-1976. II. Shigella Surveillance	53
Nguyen, T. K., Milch, H.: Klebsiella and Enterobacter Strains Derived from Hospital Infections. I. Correlation between Species, Phage Type and Antibiotic Sensitivity	67
Antoni, F., Jobbágy, A., Puskás, M.: Surface Immunoglobulins of Tonsil Lymphocytes	83
Anderlik, P., Szeri, I., Bános, Zs., Wessely, M., Radnai, B.: Effect of <i>Bordetella pertussis</i> Vaccine on Neonatally Thymectomized Mice	91
Rozgonyi, F., Biacs, P., Szűha, K., Kiss, J.: Effect of Methicillin on the Fatty Acid Composition of Phospholipids in Methicillin Sensitive <i>Staphylococcus aureus</i>	97
Csiszár, K., Lányi, B.: Proticine Typing of Serologically Defined <i>Proteus</i> Strains	111
Czirók, É., Csík, M., Börzsönyi, M.: Incidence in Enteritis of <i>Enterobacteriaceae</i> Isolates Possessing Human Colonization Factor Antigen. New Human Colonization Factors	119
In memoriam György Ivánovics	129

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GROWTH OF BACTERIA AND YEAST ON ENZYMICALLY DEGRADED ALKALI TREATED RICE AND WHEAT STRAWS

J. K. GUPTA, C. K. SHIRKOT and S. DHAWAN

Department of Microbiology, Panjab University, Chandigarh, India

(Received November 20, 1979)

The unconcentrated enzyme filtrate of *Trichoderma viride* QM 9414, giving 80 units/ml CMCase activity was used to saccharify rice and wheat straw at 45 °C. The conversion to sugar was 18.6 and 15.4% in 24 h, respectively. Pretreatment of the two materials for enzymatic saccharification showed that alkali treatment was more effective than peracetic acid treatment. Delignification by alkali treatment increased the enzymic saccharification of both materials to about 70%. The optimum conditions for delignification were with autoclaving at 120 °C for 30 min with 2% NaOH. The five bacterial species *Escherichia coli*, *Pseudomonas aeruginosa*, *Staphylococcus aureus*, *Lactobacillus acidophilus* and *Bacillus megaterium*, and the yeast *Saccharomyces cerevisiae* grew very well on enriched hydrolyzates of rice and wheat straws. Even non-enriched straw hydrolyzates supported better growth of *L. acidophilus*, *B. megaterium* and *E. coli* on rice straw than the enriched synthetic medium containing equivalent glucose. *S. cerevisiae* grown in shake flasks containing 25 ml of enriched rice and wheat straw hydrolyzates yielded 0.595 g and 0.450 g of dry cells, respectively. The corresponding yield was 0.396 g from enriched synthetic medium containing equal amounts of glucose.

Cellulose is the most abundant of all renewable organic compounds in nature and there is continued interest in its biodegradation to glucose for meeting energy and feed/food requirements. The process will, however, become economical only if we possess a reactive cellulose and a very active cellulase preparation. A search for the organisms producing highly active cellulase has led to the discovery of cellulolytic fungi like *Trichoderma viride*. Mutants of this organism [1] have yielded highly cellulolytic enzyme preparations. *T. viride* QM 9414 and QM 9123 mutants of QM 6a were thought to be best organisms until MONTENECOURT and EVELEIGH [2] mutated *T. viride* QM 6a to produce organisms with still higher activity. The new organism has been termed *Trichoderma reesei* NG-14.

The other aspect, i.e. the preparation of reactive cellulose, is an important priority and this study attempts to standardize the conditions of the chemical modification of the raw materials to make them more reactive to the enzymic attack. The rice and wheat straws chosen for this study are most common farm wastes or surplus materials. Most straws are known to contain 30–44% cellulose [3]. The exposed cellulosic material after pretreatment of these straws was subjected to enzymic hydrolysis and the hydrolyzates obtained were used for growth of common bacteria and yeast species.

Materials and methods

Enzyme preparation. A comparison of the enzyme producing capacity of some of the standard strains of the cellulolytic fungi was done with strains isolated in our laboratory. The organisms were grown under conditions described earlier [4] using CP-60 cellulose as the carbon source (a gift of Cellulose Products of India, Ahmedabad, India). *T. viride* QM 9414 supplied by Dr. M. MANDELS (U.S. Army Natick Laboratories, Massachusetts, U.S.A.) was found to produce maximum cellulase (Cx) giving 80 units of CMCase activity on carboxymethyl cellulose (CMC-7MT; medium viscosity; degree of substitution, 0.5). CMC-7MT was kindly supplied as a gift sample by Hercules Incorporated, Wilmington, Delaware, U.S.A. The enzyme preparation was used in the experiments on saccharification of rice and wheat straws.

Pretreatment of rice and wheat straws. (a) *Preparation of the materials.* The two types of straw were dried in sun and then in the oven at 60 °C for 48 h. The dried material was ground and sieved through the Indian Standard Institute (ISI) mesh to obtain the -15+10 fraction. The powders so prepared were separately treated with peracetic acid and alkali.

(b) *Treatment with peracetic acid* [5]. Peracetic acid was prepared by mixing acetic anhydride and 30% hydrogen peroxide at a ratio of 1:1. One gram of material was suspended in 18 ml of peracetic acid and autoclaved at 121 °C for one hour. The contents were filtered, washed acid free, oven dried at 60 °C, and pulverized.

(c) *Treatment with sodium hydroxide* [6]. The powdered straws were autoclaved with one percent sodium hydroxide. The effect of varying concentration of alkali (0.5, 1, 2, 4 and 6%), autoclaving time (15, 30, 45 and 60 min) and treatment temperature (50, 80, 100 and 120 °C) on delignification of the two materials was studied.

Saccharification of the treated materials. The pretreated materials were subjected to enzymic hydrolysis [4]. Four ml of the enzyme filtrate were incubated with 300 mg of the powdered cellulosic materials and 3.0 ml of citrate buffer (0.055 M, pH 5.0) in 30 ml test tubes at 45 °C for 24 or 48 h. The reducing sugars formed were estimated by the dinitrosalicylic acid procedure [7].

Growth of baker's yeast and bacteria on rice and wheat straw hydrolyzates. Four different media were prepared using wheat and rice straw hydrolyzates as follows. (a) Wheat hydrolyzate medium containing products of enzymic hydrolysis of wheat straw. (b) Enriched wheat hydrolyzate medium consisting of media (a) and 0.3% each of peptone and yeast extract. (c) Rice hydrolyzate medium containing products of enzymic hydrolysis of rice straw. (d) Enriched rice hydrolyzate medium consisting of media (c) plus 0.3% each of peptone and yeast extract.

For comparison, two synthetic media containing 4.0 and 3.8% glucose equivalent to total reducing sugars in rice and wheat straw hydrolyzates, respectively, were prepared. Two enriched synthetic media were also prepared; they contained 0.3% each of peptone and yeast extract and the glucose level was 4.0% in one and 3.8% in the other. The pH of these media was adjusted to 7.0 for bacteria and to 5.0 for yeast.

Three ml of each of the media were dispensed into small test tubes (1×10 cm), autoclaved at 120 °C for 20 min and inoculated with the overnight culture of five bacterial species (*E. coli*, *P. aeruginosa*, *S. aureus*, *L. acidophilus* and *B. megaterium*) and a yeast (*S. cerevisiae*). The growth in the tubes was measured by reading absorbance on Spectronic-20 at 600 nm after 8, 16, 24, 36, 48, 72 and 96 h incubation.

S. cerevisiae was grown in large volumes. Twenty-five ml of the enriched hydrolyzates were transferred to Erlenmeyer flasks and inoculated with a loopful of *S. cerevisiae* after autoclaving at 120 °C for 20 min. The flasks were incubated in a rotary shaker at 28 °C for four days. At the end of the incubation the contents of the flasks were centrifuged and wet and dry weight of the cells was recorded. For taking the dry weight the cells were transferred in small aluminium wells and dried at 50 °C to constant weight. The supernatant was analyzed for residual sugars. The growth obtained on the hydrolyzates was compared with the growth on synthetic media enriched with 0.3% peptone and yeast extract.

Results and discussion

Enzymic degradation of peracetic acid and alkali treated straws. Results of saccharification are in Fig. 1. Alkali treatment for 1 h resulted in a considerable increase in the enzymatic degradation of rice and wheat straws.

In 24 h these materials were degraded up to 66.5% and 59.5%, respectively. Even autoclaving with water had improved the susceptibility of the materials and in the case of wheat straw saccharification increased from 15.4 to 22.2%.

TOYAMA and OGAWA [8] found alkali treatment to be ineffective in case of woody wastes; this was, however, contradicted by GOLDSTEIN and VILLAR-

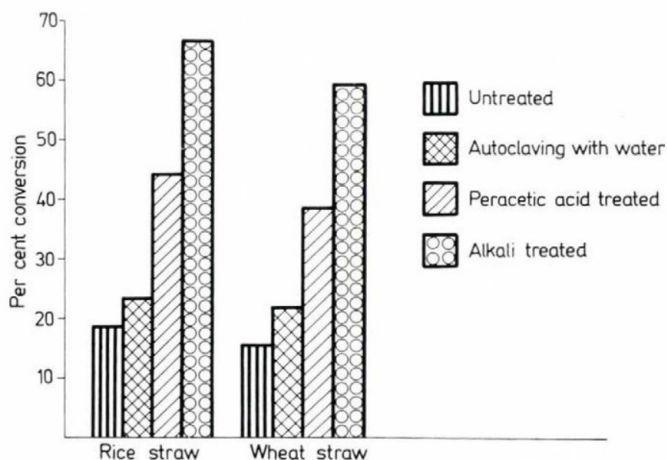


Fig. 1. Effect of pretreatment on enzymic degradation of rice and wheat straws

ROAL [9] and DIETRICH and HONNECKE [10]. Alkali treatment has been known to increase the susceptibility of rice straw to enzymic attack [6, 11-13].

The effect of pretreatment was most prominent when the straws were treated with alkali. The two materials were further treated with alkali at different concentrations, at different temperatures and for different time intervals.

Degradation of alkali treated straws. Effect of NaOH concentration. Rice and wheat straw powders obtained after treatment with 0.5, 1, 2, 4 and 6% sodium hydroxide autoclaved for 1 h, were incubated with enzyme at 45 °C for 24 h.

It was evident (Table I) that both substrates showed maximum saccharification on 2% NaOH treatment. At this level, the degradation of the two straws increased to 74.2 and 68.8% compared to 52.2 and 42.0%, respectively, with 0.5% NaOH treatment. At higher concentrations of alkali, the saccharification was detrimentally affected.

TOYAMA and OGAWA [6] delignified rice straw by autoclaving with 1% NaOH at 120 °C for one hour. Under identical conditions, 4% NaOH in a ratio of 1 : 6 was found the best for delignification of rice straw and rice hulls [14]. IOSHI and PARSHAD [15] delignified sugarcane bagasse by heating to 100 °C in 0.5 N NaOH at a ratio of 1 : 20.

Table I*Enzymic saccharification of rice and wheat straws pretreated with alkali at 120 °C for 1 h*

NaOH treatment, %	Enzymic saccharification			
	Rice straw		Wheat straw	
	total sugars, mg	saccharification, %	total sugars, mg	saccharification, %
0.5	156.6	52.2	126.0	42.0
1.0	201.6	67.2	186.2	62.06
2.0	222.6	74.2	206.5	68.8
4.0	190.4	63.4	178.5	59.5
6.0	140.0	46.6	130.9	43.6

Reaction mixture: enzyme filtrate (80 units/ml), 4.0 ml; raw cellulose, 300 mg; citrate buffer (0.055 M, pH 5.0), 3 ml; temp. 45 °C, incubation period 24 h

$$\text{Percent saccharification} = \frac{\text{total sugars formed}}{\text{weight of substrate}} \times 100$$

Effect of treatment time. Two percent NaOH being the optimum concentration, delignification was continued at this concentration by autoclaving the two straw powders for time intervals of 15, 30, 45 and 60 min. The treated materials were subjected to enzymic saccharification and it was found (Table II) that sugar formation increased with pretreatment time from 15 to 30 min by 61.5 to 73.5% with rice straw and 56.0 to 69.06% with wheat straw, but saccharification did not increase further on increasing the time of treatment.

Table II*Enzymic saccharification of rice and wheat straws pretreated with 2% alkali at 120 °C for different time periods*

Pretreatment time, min	Enzymic saccharification			
	Rice straw		Wheat straw	
	total sugars, mg	saccharification, %	total sugars, mg	saccharification, %
15	184.8	61.6	168.0	56.0
30	220.5	73.5	207.2	69.06
45	222.6	74.2	208.6	69.5
60	214.9	71.6	208.6	69.5

Reaction mixture: same as in Table I

Effect of treatment temperature. Rice and wheat straw with 2% alkali were exposed to 50, 80, 100 and 120 °C for 30 min (Table III) and subsequently subjected to enzymic treatment.

Table III

Effect of pretreatment temperature on enzymic saccharification of rice and wheat straws treated with 2% NaOH for 30 min

Pretreatment temperature, °C	Enzymic saccharification			
	Rice straw		Wheat straws	
	total sugars, mg	saccharification, %	total sugars, mg	saccharification, %
50	140.0	46.6	130.2	43.4
80	164.5	54.8	154.0	51.3
100	196.6	65.6	199.0	66.3
120 (autoclaving)	225.4	75.1	210.0	70.0

Reaction mixture: same as in Table I

The extent of saccharification of the substrates increased gradually with the increase in temperature of alkali pretreatment. It was maximum (75.1%) at 120 °C and minimum at 50 °C (46.6%) in the case of rice straw. Similar results were obtained with wheat straw. It seems that autoclaving temperature is most suitable for increased penetration of the alkali, resulting in higher delignification and subsequent saccharification.

Growth studies on rice and wheat straw hydrolyzates. The optimum conditions of alkali treatment reported above were, 2% NaOH at 120 °C for 30 min. Rice and wheat straws pretreated under these conditions and subjected to enzymic saccharification for 48 h gave a hydrolyzate which contained 4% and 3.8% reducing sugars, respectively. The enriched and non-enriched media based on these hydrolyzates and synthetic media with equivalent glucose were inoculated with five bacterial and one yeast species and growth was measured periodically.

It was observed (Figs 2, 3 and 4) that the hydrolyzate enriched with peptone and yeast extract allowed best growth of all the organisms tested. On the other hand, the growth was slowest in non-enriched synthetic medium containing only glucose. The growth on enriched hydrolyzate medium was probably enhanced due to the known and unknown growth promoting factors present in the enzyme filtrate. The enzyme filtrate might have contained some unutilized and liberated amino acids, unutilized minerals and other nutrients which originated from the enzyme production medium. These resulted in an

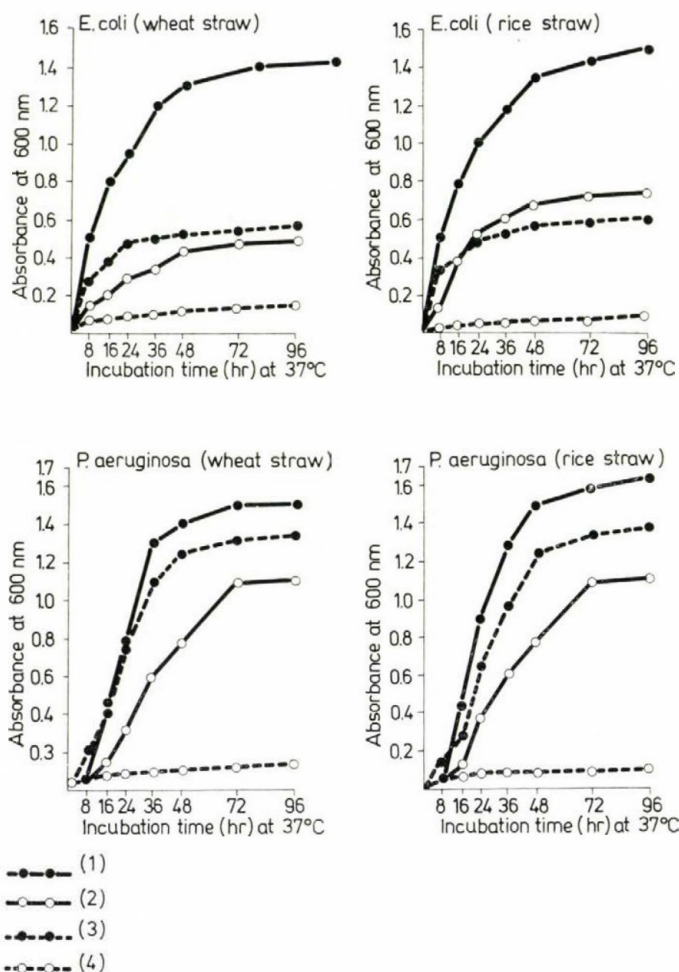


Fig. 2. Growth of *E. coli* and *P. aeruginosa* on wheat and rice hydrolyzates. (1) Enriched hydrolysates; (2) hydrolysates; (3) enriched synthetic medium; (4) synthetic medium

enriched hydrolyzate further enriched with peptone and yeast extract. On the other hand, synthetic medium containing only glucose as carbon source was apparently not able to support the growth in the absence of all the other growth promoting factors.

It was interesting that even non-enriched hydrolyzate medium was better than enriched synthetic medium for the growth of *E. coli* on rice straw hydrolyzates, and of *L. acidophilus* and *B. megaterium* on both hydrolyzates. Lactobacilli are known to require a large number of growth factors in the medium [16]. This clearly indicates that the growth requirement of these organisms was met by the pentoses and hexoses liberated through the hydrol-

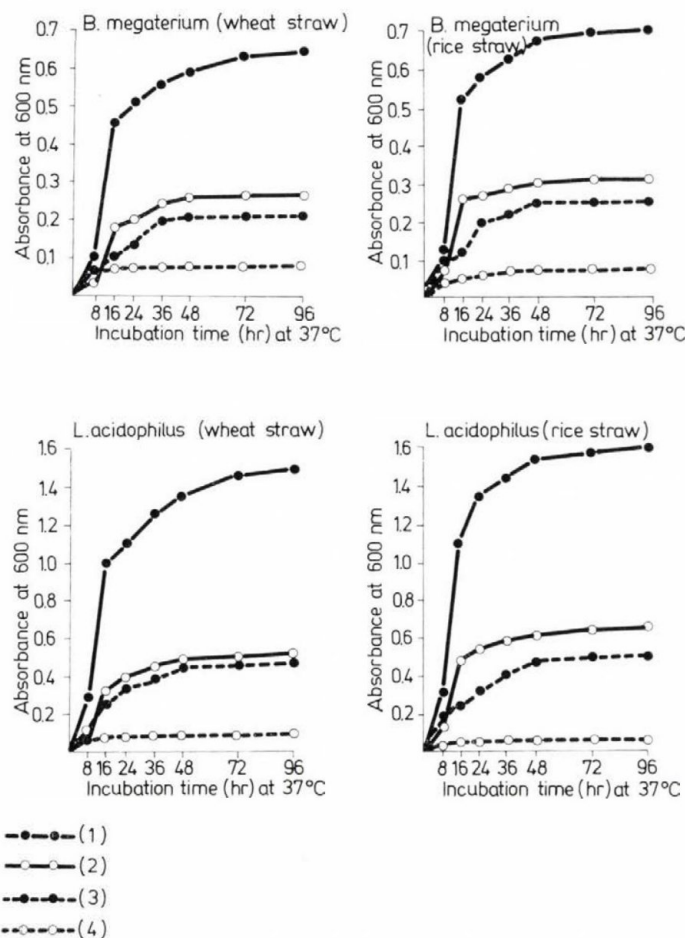


Fig. 3. Growth of *B. megaterium* and *L. acidophilus* on wheat and rice straw hydrolyzates. For legends see Fig. 2

ysis of celluloses and hemicelluloses present in straws and other nutrients present in the hydrolyzates. Characterization of the nutrients other than sugars needs further investigation.

E. coli in wheat hydrolyzate and *P. aeruginosa* and *S. aureus* on both rice and wheat hydrolyzates, and *S. cerevisiae*, showed better growth on enriched synthetic medium than on non-enriched hydrolyzates. GULATI [17] reported better growth of *Rhizobium* species on pea husk and water-hyacinth hydrolyzates than on yeast extract-mannitol (YEM) synthetic medium.

Production of yeast on straw hydrolyzates. The enriched straw hydrolyzates were tried for growing *S. cerevisiae* in large volumes. The growth so obtained was compared with the yields from the synthetic media. The data

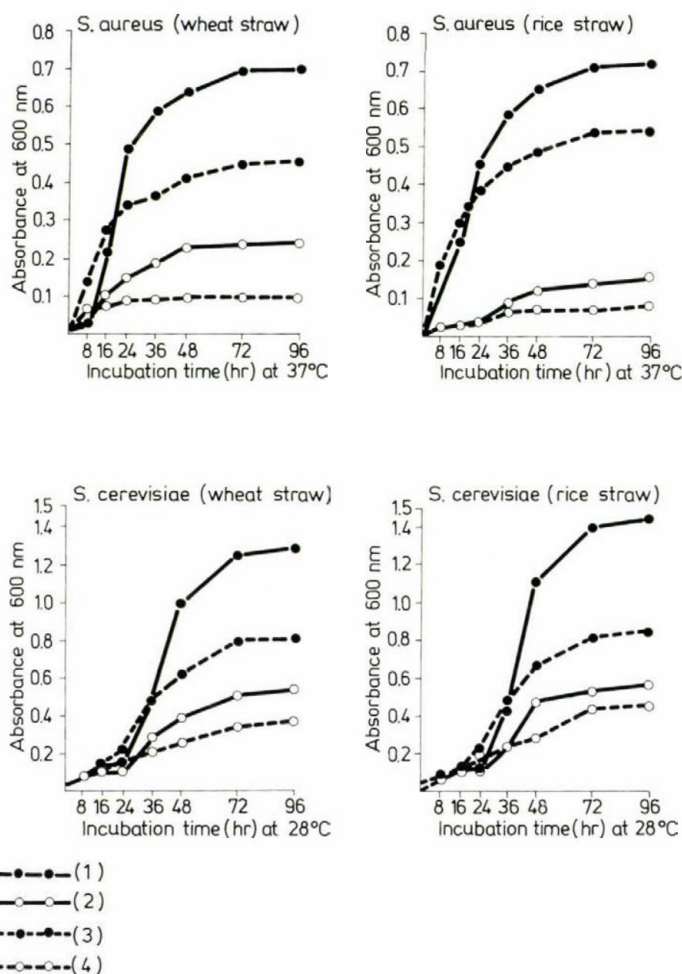


Fig. 4. Growth of *S. aureus* and *S. cerevisiae* on wheat and rice straw hydrolyzates. For legends see Fig. 2

on the wet and dry weight of yeast cells and the initial and final sugar concentrations are presented in Table IV.

Enriched rice straw hydrolyzate was the best medium for the production of yeast giving 0.595 g of dry cells vs. 0.450 g in enriched wheat straw hydrolyzate and 0.396 g in enriched synthetic medium. Clearly, some sugars or oligosaccharides present in the rice straw hydrolyzate were responsible for the better yeast growth. The good growth shown by enriched hydrolyzates was also reflected in the low levels of the residual sugars. The enriched synthetic medium contained 0.036% of residual sugars compared to 0.07 and 0.12% in enriched rice and wheat hydrolyzates, respectively. The lowest value

Table IV
Production of baker's yeast on rice and wheat straw hydrolyzates

Growth medium	Initial sugar concentration, %	Wet weight of yeast, g	Dry weight of yeast, g	Final sugar concentration, %
Enriched* rice hydrolyzate	4.0	2.462	0.595	0.07
Rice hydrolyzate	4.0	1.050	0.270	0.9
Enriched* wheat hydrolyzate	3.8	2.105	0.450	0.12
Wheat hydrolyzate	3.8	0.945	0.258	1.12
Enriched* synthetic medium	4.0	1.353	0.396	0.036
Synthetic medium	4.0	0.440	0.110	0.1

* Enrichment was done by adding 0.3% each of peptone and yeast extract

obtained on enriched synthetic medium was due to the fact that the only sugar present in it, i.e. glucose, was completely fermentable. On the other hand, the straw hydrolyzates contained pentoses like xylose and arabinose which are not utilized by *S. cerevisiae*.

TOYAMA and OGAWA [6] prepared hydrolyzate from rice straw with Meicelase (cellulase) enzyme and supplemented it with nutrients of Hayduck medium. From 100 ml of the hydrolyzate containing 10.91% of sugar they obtained 0.8 g of freeze dried yeast cells. Similarly, the hydrolyzates containing 3.56 and 3.50% initial sugars gave 1.05 g and 0.98 g yields for two strains of *Candida utilis*. We have obtained much higher yields of *S. cerevisiae* from rice and wheat straw hydrolyzate containing nearly the same amount of sugar but enriched with peptone and yeast extract.

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SOIL MICROBIOLOGICAL ASSESSMENT OF TOXICITY OF ALKANOATE, AMIDE AND OTHER HERBICIDES

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The toxicity of alkanolate (2,4-D and MCPA), amide (CDAA, alachlor, propachlor and diphenamide) and some other herbicides (trifluralin, dalapon, bromacil, lenacil) was studied on microorganisms and nitrification of smolnitsa and alluvial soils. Kruglov's coefficient was used for the purpose; it characterizes the inhibitory effect of pesticides from the point of view of soil microbiology. The results showed that the herbicides studied inhibit nitrification and the amount of soil bacteria, actinomycetes and microscopic fungi. Herbicide toxicity is smaller with smolnitsa, which is related to a greater adsorption of the preparations.

Long-term studies have been carried out in the last few years to establish the effect of pesticides on useful soil microorganisms [1], which came as a logical consequence of the increasing use of pesticides in agriculture. So far, no common criterion has been accepted to assess the toxicity of herbicides. That has embarrassed methodology and interpretation, in particular, of the results of studies carried out under different conditions.

The purpose of this work was to establish the harmful effect of some widely applied herbicides on soil microorganisms with the aid of a criterion suggested by KRUGLOV [2].

Materials and methods

The toxicity has been studied of alkanolate (2,4-D and MCPA), amide (alidochlor, alachlor, propachlor, diphenamide) and other herbicides (trifluralin, dalapon, bromacil, lenacil and dichlobenil); see Table I. *

The effect of these herbicides has been tested on the quantity of soil bacteria (on meat-peptone agar), actinomycetes (on starch-ammonia agar), microscopic fungi (on Chapek's medium; see Table II) and nitrifying activity [3, 4]. Smolnitsa (from the village of Bozhourishte, district of Sofia) and alluvial soil (from Kostinbrod) were used in the study.

Assessment of the toxic effect on nitrification and of the microorganisms in the same soils was done by KRUGLOV's equation [2]:

$$Cs = \frac{IC_{50} \text{ or } QC_{80}}{PC_{min}}$$

where Cs is the coefficient of security; IC_{50} or QC_{80} are concentrations of the herbicide reducing the intensity of microbiological processes and the contents of microorganisms by 50% and 80% respectively; and PC_{min} is the minimum concentration of the herbicide applied. Herbicide toxicity is determined according to the value for Cs . If Cs is smaller than 1, the preparation

Table I
Herbicides used in this study

Code name	Proprietary name	Chemical name	Manufacturer	Country
2,4-D	Spritzhormit	(2,4-dichlorophenoxy)acetic acid	Elektrochemisches Kombinat, Bitterfeld	G.D.R.
MCPA	Dilcotex	(4-chloro-o-tolyl)oxy acetic acid		Czechoslovakia
Diphenamide	Dimid	N,N-dimethyl-2,2-diphenylacetamide	Elanco	U.S.A.
Alidochlor (CDAA)	Randox	N,N-diallyl-2-chloroacetamide	Monsanto	U.S.A.
Alachlor	Lasso	2-chloro-2,6-diethyl-N-(methoxymethyl)acetamide	Monsanto	U.S.A.
Propachlor	Ramrod	2-chlor-V-isopropyl-N-phenylacetamide	Monsanto	U.S.A.
Dichlobenil	Casoron	2,6-dichlorobenzonitrile	Philips Duphar	Netherlands
Trifluralin	Treflan	-trifluoro-2,6-dinitro-N,N-dipropyltoluidine	Elanco	U.S.A.
Dalapon	Basfapon	2,2-dichloropropionic acid	BASF	West-Germany
Lenacil	Vensar	cyclohexyl-5,6-trimethylenuracil	DuPont	U.S.A.
Bromacil	Hyvar X	5-bromo-3-sec-butyl-6-methyluracil	DuPont	U.S.A.

is toxic; if it is from 1 to 10, it is an inhibitor; if it is between 10 and 100, it is a weak inhibitor; while if the Cs is over 100, it is innocuous (nontoxic). IC₅₀ and QC₈₀ are graphically determined on the basis of analyses on nitrification and the quantity of microorganisms at gradually increasing concentrations of the herbicides. The experiments were carried out under laboratory conditions at 27 °C and 60–70% soil moisture water holding capacity (WHC) for a period of 10 days. The quantity of nitrate was determined by distillation.

Results and discussion

The effect of the above herbicides at a concentration of 80 mg/kg soil pointed to a decrease of the intensity of nitrification (Fig. 1), which was most marked with alidochlor and propachlor, and the amount of microorganisms (Table III) which was greatest with fungi, caused by alidochlor and dichlobenil. The approximately equal quantity of bacteria and actinomyces in the control shows that the quantity of micromineralizing organic matter in the studied plots was very low. Diphenamide and trifluralin are characterized by the smallest effect on nitrification, while diphenamide, dalapon, and propachlor exert the weakest effect on microorganisms. The effect of all the herbicides mentioned above is expressed but interpretation of the results obtained for a comparative assessment of the toxicity of the respective prep-

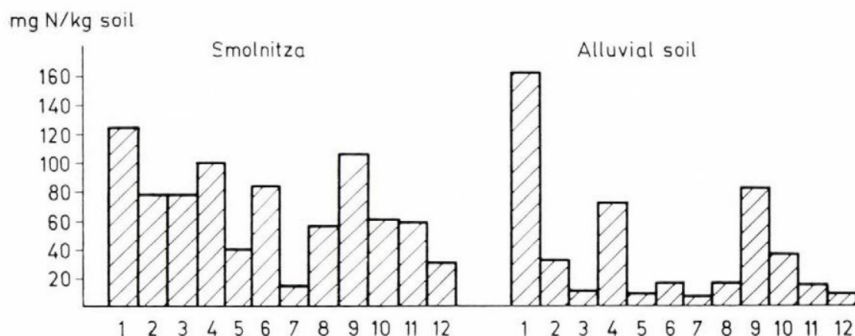


Fig. 1. Influence of the herbicides on nitrification. 1 = Control; 2 = 2,4-D; 3 = MCPA; 4 = Diphenamide; 5 = CDAA; 6 = Alachlor; 7 = Propachlor; 8 = Dichlobenil; 9 = Trifluralin; 10 = Dalapon; 11 = Lenacil; 12 = Bromacil

Table II
Ingredients of plate media

Meat-peptone agar		Starch-ammonium agar		Media of Chapek	
meat infusion	1 litre	(NH ₄) ₂ SO ₄	1 g	NaNO ₃	2 g
NaCl	5 g	K ₂ HPO ₄	1 g	KH ₂ PO ₄	1 g
peptone	10 g	MgSO ₄	1 g	KCl	0.5 g
agar	20 g	NaCl	1 g	MgSO ₄	0.5 g
pH 7		CaCO ₃	3 g	FeSO ₄	0.01 g
		starch	10 g	sucrose	30 g
		agar	20 g	agar	20 g
		water	1 litre	pH 4	
		pH 7			

Physico-chemical indices of the soils

Soil	Silt content (soil particles <0.001 mm)	pH in KCl	Humus, %	Carbonates	Sorption capacity, mg/100 g soil
smolnitsa	52	5.7	2.75	0	50.88
alluvial soil	0.9	6.6	0.31	0	21.31

arations is more precisely determined by the method of Kruglov. The results obtained by that method are given in Table IV.

The data presented show that the value of Cs for most of the herbicides used was over 10. This means that they are weak inhibitors of nitrification, as well as of the development of microorganisms. Some of them are even

Table III

Inhibition of nitrification and microorganisms in smolnitsa and alluvial soils as affected by herbicides used at 80 mg/kg

Soil and herbicide	Bacteria millions/g soil – standard deviation	Actinomycetes millions/g soil – standard deviation	Fungi thousands/g soil – standard deviation
Smolnitsa			
Control	16.6 – 0.3	16.8 – 0.4	120 – 0
2,4-D	13.0 – 0.5	13.9 – 0.5	90 – 5
MCPA	12.5 – 0.63	13.3 – 0.15	80 – 0
Diphenamide	16.0 – 0	15.2 – 0	110 – 25
CDAA	9.0 – 0.5	9.3 – 0.35	40 – 0
Alachlor	12.1 – 0.5	12.8 – 0.4	90 – 5
Propachlor	14.5 – 0.25	15.8 – 0.4	100 – 0
Dichlobenil	13.0 – 0.1	11.8 – 0.5	50 – 5
Trifluralin	14.0 – 0	15.8 – 0.2	100 – 0
Dalapon	15.0 – 0.5	15.7 – 0.16	100 – 0
Lenacil	10.8 – 0.4	11.2 – 0.4	60 – 0
Bromacil	16.5 – 0.24	16.7 – 0.15	100 – 0
Alluvial soil			
Control	14.6 – 0.3	14.0 – 0	70 – 5
2,4-D	9.9 – 0.5	11.9 – 0.6	40 – 0
MCPA	9.5 – 0.24	11.0 – 0.5	30 – 3.67
Diphenamide	14.0 – 0	12.5 – 0.24	60 – 0
CDAA	8.0 – 0	7.4 – 0.3	10 – 3.67
Alachlor	9.8 – 0.1	10.2 – 0.1	40 – 3.67
Propachlor	12.0 – 0	12.7 – 0.34	50 – 5
Dichlobenil	9.8 – 0.1	9.6 – 0.6	10 – 3.67
Dalapon	13.0 – 0.5	12.6 – 0.7	50 – 5
Trifluralin	10.5 – 0.24	13.0 – 0.59	50 – 5
Lenacil	8.6 – 0.3	9.4 – 0.6	20 – 0
Bromacil	14.4 – 0	13.9 – 0.65	60 – 10

innocuous as regards some groups of microorganisms and nitrification. The Cs values were found highest for diphenamide, 2,4-D and MCPA; and lowest, for alidochlor and dichlobenil. Therefore, the latter could be considered the most dangerous preparations.

As regards the soils studied, as they are characterized by significant physicochemical differences, a certain reduction of the indices must be made

Table IV

Toxic effect of herbicides on nitrification and on microflora on smolnitsa and alluvial soils

Soil and herbicide	Dose mg/kg (in practical application)	Nitrification		Bacteria		Actinomycetes		Microscopic fungi	
		IC ₅₀	Cs	QC ₅₀	Cs	QC ₈₀	Cs	QC ₈₀	Cs
		mg/kg							
Smolnitsa									
2,4-D	1.2	216	179	147	123	184	157	128	106
MCPA	1.2	216	179	130	107	153	127	96	80
Diphenamide	3.2	420	131	864	270	333	104	380	119
CDAA	4.0	115	28	70	17	71	18	48	12
Alachlor	3.0	250	83	117	39	133	44	128	43
Propachlor	3.3	90	27	251	76	533	161	192	58
Dichlobenil	4.0	145	36	147	37	107	26	54	13
Trifluralin	0.5	57	114	203	407	484	968	192	384
Dalapon	1.8	160	89	320	177	484	269	192	107
Lenacil	0.6	151	195	91	142	96	149	64	106
Bromacil	2.0	105	52	5471	2285	2666	1333	192	96
Alluvial soil									
2,4-D	1.2	99	82	99	83	160	133	75	62
MCPA	1.2	86	71	91	76	148	124	56	46
Diphenamide	3.2	133	42	761	237	296	93	223	70
CDAA	4.0	84	21	70	18	68	17	37	9
Alachlor	3.0	89	30	98	32	118	40	75	25
Propachlor	3.3	82	25	178	54	344	104	112	34
Dichlobenil	4.0	89	22	97	24	102	25	37	9
Trifluralin	0.5	160	320	117	228	444	888	112	34
Dalapon	1.8	102	57	290	161	320	177	112	34
Lenacil	0.6	87	145	78	121	97	151	44	74
Bromacil	2.0	84	42	2285	1143	4000	2000	223	70

IC₅₀ and QC₈₀ = concentrations of the herbicides reducing by 50% and 80% respectively the intensity of nitrification and the quantity of microorganisms in soils; Cs = coefficient of security.

for alluvial soil. Pesticide toxicity may occur more often in that soil than in smolnitsa. The high absorption of the preparations in smolnitsa, related to the considerable amount of clay minerals in that soil, is a factor determining their innocuous effect from the microbiological point of view.

The coefficient of security (Cs) was over 10 for most of the herbicides tested and that is why they are characterized by Kruglov as weak inhibitors. According to the same criterion, alidochlor and dichlobenil are considered to be more toxic (Cs over 10) but only as far as fungi are concerned. The latter are more sensitive than the rest to all the herbicides studied and, therefore, the coefficient has a smaller value for them. The toxic effect of herbicides is greater in alluvial soils than in smolnitsas, where the strong adsorption causes a certain neutralization of the effect. Of the herbicides analysed, alidochlor and dichlobenil are comparatively more dangerous from the point of view of environmental pollution.

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CONNECTIONS BETWEEN THE HEXON POLYPEPTIDES IN THE TWO-DIMENSIONAL HEXON CRYSTALLINE ARRAY

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Fine interhexonal connections were observed in high resolution electron micrographs of tight two-dimensional hexagonal crystalline arrays. Intrahexonal connections were found among the three polypeptides constituting the hexons. The length of the interhexonal connections was 2.73 nm, and their diameter 1.2 nm; that of the intrahexonal connections were 1.05 nm and 0.75 nm, respectively. The orientation of the hexons in a two-dimensional crystalline array corresponded to a corner to edge conjunction. One side of each of the three polypeptides, constituting a triangular hexon, corresponded to the sides of that hexon; their points of contact were the corners. A tentative diagram of the molecular arrangement of hexon polypeptides and of interhexonal connections was drawn up following analysis of the electron micrographs. It showed that parallel connections run to both ends of each polypeptide of a hexon, connected to one of the end parts of the two nearest polypeptides of the next hexon, as follows from the threefold symmetry. Thus, every hexon is joined to its six neighbours by six times two parallel connections. Two pairs of parallel connections enclose an angle of approximately 60°.

Previous experiments have shown that highly purified and concentrated hexon capsomers extracted from infected cells, can be crystallized. There arise three-dimensional tetrahedral hexon crystals and tightly packed two-dimensional hexon crystalline arrays [1–3]. The hexons of the latter array form a hexagonal packing similar to the hexon capsomers in the capsid of virus particles [4, 5]. It was shown that hexons of a two-dimensional array were connected to the six nearest hexons by fine bridge-like elements.

The present report gives further information on the connections between complete hexons and shows connections between the three polypeptide subunits that build up a hexon.

Materials and methods

Purification of hexon proteins. The soluble protein components extracted from HEp-2 cells previously infected with adenovirus type 1, were separated from the virions by ultracentrifugation. The hexon capsomers were separated and purified by repeated anion exchange chromatography and gel filtration [6–8]. Protein concentration was determined by the method of LOWRY *et al.* [9].

Crystallization. The highly purified and concentrated hexon preparations were dialysed against 0.5 M acetate buffer pH 4.5 at +4 °C for 72 h, and subsequently stored in glass tubes at +4 °C, and examined by electron microscopy and light microscopy. Light microscopic observation revealed the development of three-dimensional tetrahedral crystals in some hours or days, depending on the concentration. The simultaneous development of two-dimensional mono-layer hexon crystalline arrays could be detected by electron microscopy [1–3, 10, 11].

Electron microscopic examinations. The samples were adsorbed to carbon-formvar coated grids, negatively stained with 1% uranyl acetate and examined by a JEM 100 B electron microscope at 60 kV and at a basic magnification of $\times 100\,000$. Electron micrographs were made by enlarging the electron microscopic negative films to a magnification of $\times 300\,000$ or $\times 700\,000$. In certain cases these enlarged photographs were reproduced and further magnified, and examined as a photograph or a slide. The background granulation of the sharply focussed photos made directly of the electron microscopic negative films often ruled out a detailed observation of the fine structure. Underfocussed enlargements improved the quality of the photos [12, 13] and thus, finely structured elements were easy to distinguish.

Results

Size of the interhexonal connections. In a tight two-dimensional hexagonal crystalline array there are 1.5 to 3 nm spaces between the hexons. This average size was obtained by measuring the distances in the direction of the three non-parallel hexon rows several hundred times and in different crystal-sheets. The obtained values were slightly different in the three non-parallel hexon rows in all preparations, e.g. the average distance between the hexons of the two-dimensional crystalline array in Fig. 1 was 1.75 ± 0.1 nm in the vertical hexon row, 2.75 ± 0.44 nm in the adjoining hexon row to the left in an angle of 60° and 1.5 ± 0.27 nm in the adjoining hexon row to the right; moreover, the average size of the interhexonal distances varied somewhat in different preparations [2, 11].

The enlargements of the high resolution electron micrographs (cp. Materials and methods) proved the presence of fine connections bridging the inter-

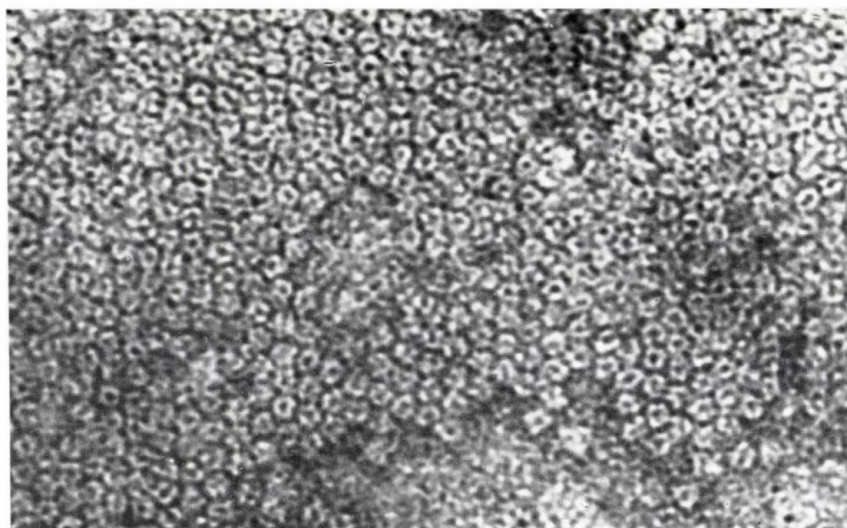


Fig. 1. Electron micrograph of a two-dimensional crystalline array, stained with uranyl acetate. Fine radial connections run between the hexons, their average size being $1.5\text{--}3\text{ nm} \times 0.5\text{--}1.5\text{ nm}$

hexonal spaces between neighbouring hexons. The length of these connections corresponded to the interhexonal distances; their diameter ranged from 0.5 nm to 1.5 nm. Interhexonal connections were shown in the virus capsid as well [12, 14].

Shape of the interhexonal connections. The interhexonal connections showed various forms. In general, like straight bridges they link the neighbouring hexons but their middle or end often become thinner (Fig. 1). Interhexonal connections deviating from the average size, being 2–3 nm in diameter, were not rare, either; these were probably double connections joined along their longitudinal axis (Fig. 2).

Number and position of the connections. The connections set out radially in several points of one complete hexon and reach the six next hexons, thus enclosing a certain angle with one another (Fig. 1). In some cases, however, two connections seem to be linked by two almost parallel elements, and three hexons by six elements. Those six connections divide into four parts the empty space enclosed by three hexons: the connections in a central position form a triangular space with an adjoining quadrangle — square or parallelepiped — to each of its sides enclosed by parallel connections. The triangular space in the middle emerges due to the V shaped lines — joining in an approximately 60° angle — described by the central connections from any hexon to its two neighbours (Fig. 3). Y-shaped connections, with three 120° angles, linking the three next hexons are also frequent; they have probably evolved out of the linkage of partially retracted central connections that normally form a triangle. Their thickness is generally above the average. They divide the space enclosed by the three next hexons into three, almost oval parts (Fig. 4). There may be no evidence of a connection between two next hexons (Fig. 5) but, in general, 6–10 connections could be discovered between any hexon and its six nearest neighbours (Fig. 6).

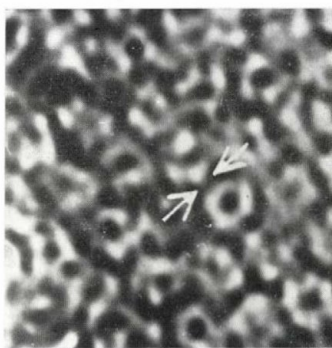


Fig. 2. The arrows point to an unusually wide connection between two hexons (30 nm). It is probably a linkage of two parallel connections joined along their longitudinal axes

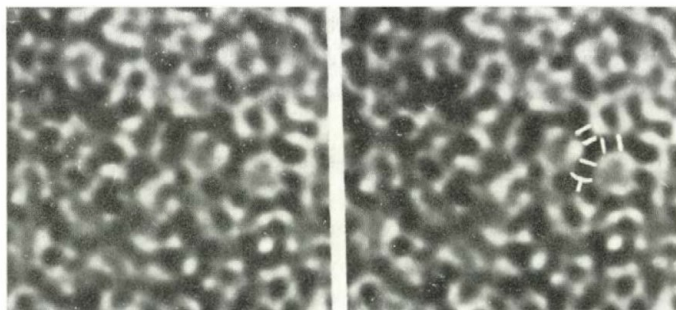


Fig. 3. Three pairs of parallel connections (marked in the photo to the right) divide the space among three neighbouring hexons into four parts: the central area is triangular. Two pairs of connections enclose angles of approximately 60° with one another

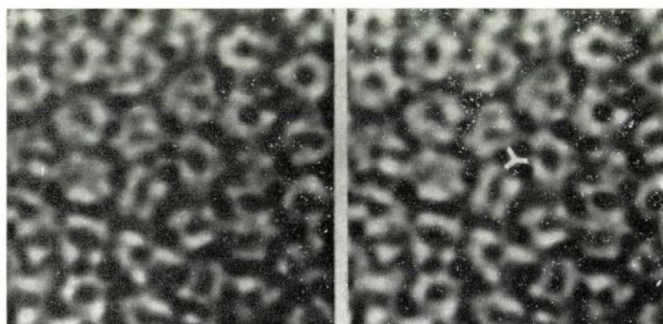


Fig. 4. A Y-shaped connection (marked in the picture to the right). It divides the space enclosed by three neighbouring hexons into three parts. The three connections forming a triangular space as shown in Fig. 3, had probably retracted and joined in the middle

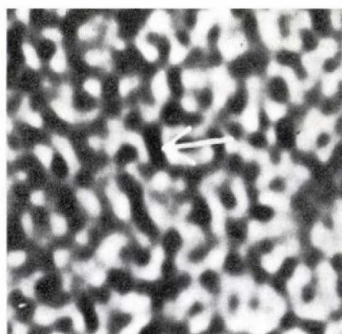


Fig. 5. The arrow points to an interhexonal space with no visible connections

Intrahexonal connections. A complete hexon capsomer consists of three polypeptide subunits in a threefold symmetry structure [7, 15–18]. In samples stained negatively with uranyl acetate or any other negative stain, the three main units that build a hexon, are seldom clearly discernible, due to a shift

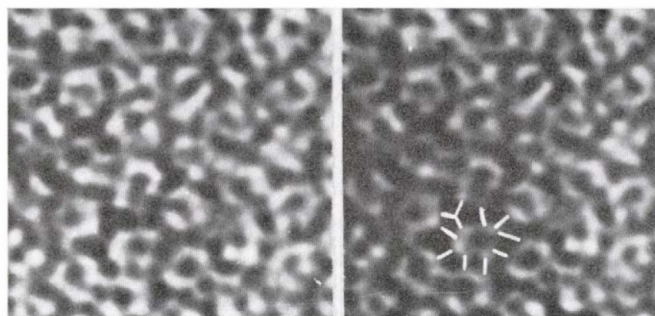


Fig. 6. Hexon in central position, linked to its neighbours by ten connections (marked in the picture to the right). The Y-shaped connection links the central hexon to two other ones which explains why it can be counted as more than one connection

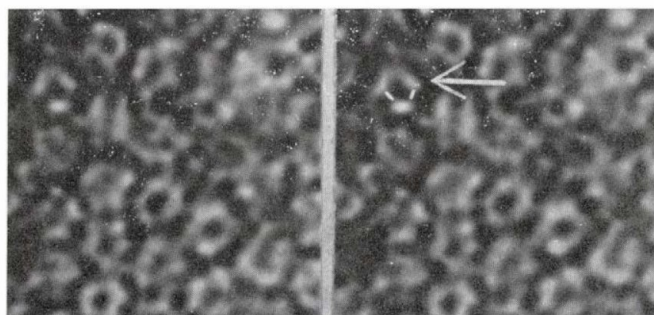


Fig. 7. A hexon with its three, clearly distinguishable constituent polypeptides. Intra-hexonal connections (marked in the photo to the right) linking one polypeptide to the others, are well visible

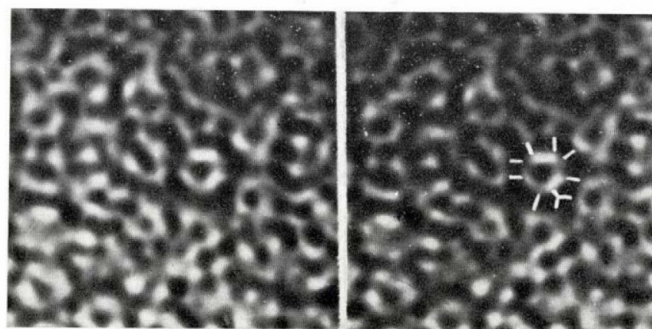


Fig. 8. The interhexonal connections run from each end of the three main polypeptides, constituting the hexon, to the neighbouring hexons

in the position or bend of one end of each polypeptide in relation to the other end and also to staining. Thus, hexons might seem to consist even of six polypeptides [16]. Nevertheless, there were in the uranyl acetate stained two-dimensional crystal sheets several hexons which clearly displayed three basic

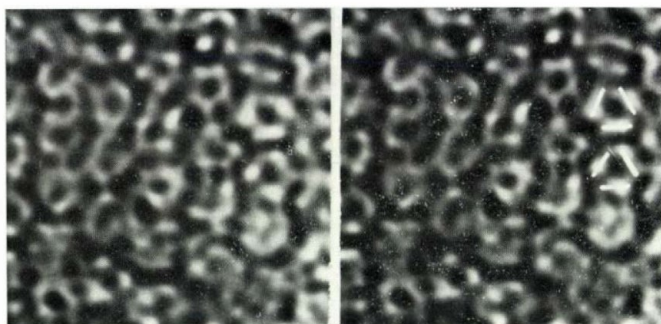


Fig. 9. Two neighbouring hexons join in a corner to edge orientation to each other (marked in the photo to the right). The sides of the triangular hexons are probably the longer sides of the polypeptides, the corners of the hexons are probably where two polypeptides meet

units. Fine connections linking these main structural polypeptides could often be observed. The intrahexonal connections in Fig. 7 are approximately 0.75 nm thick and 1.05 nm long. By joining the three polypeptides they build the complete hexon with a hole or a channel in its centre. That explains why hexons display an annular shape in negatively stained preparations. The polypeptides in a hexon seem to be linked by doubled intrahexonal bridges, though the possibility of double connections cannot be excluded. Sometimes it can be seen that the inter- and intrahexonal elements extend between the ends of polypeptides either of two neighbouring hexons or within one hexon (Fig. 8). The main polypeptides of a hexon form a parallelepiped, sometimes slightly bent, and their joinings indicate the almost triangular shape of the hexon. The corners of the triangle are probably where two polypeptides meet, its sides being the longer side of the polypeptides. In any two neighbouring hexons the polypeptides are oriented in such a way that in regular cases they meet corner to edge (Fig. 9).

Discussion

On the basis of our observations and the existence of the threefold symmetry axis of hexons [15, 16, 19] we constructed a tentative diagram of the molecular arrangement of the tight two-dimensional hexon crystalline array (Fig. 10). Part *a* of the diagram represents the respective positions of the three polypeptides within a hexon that they constitute, and the mutual orientation of the hexons in a two-dimensional crystalline array. In this structure each hexon has the same orientation and is — as due to the hexagonal packing and the threefold symmetry axis — in an equivalent environment. The sides of one polypeptide, i.e. the sides of the triangular hexon, are always nearest to the ends of the two polypeptides of a neighbouring hexon. This

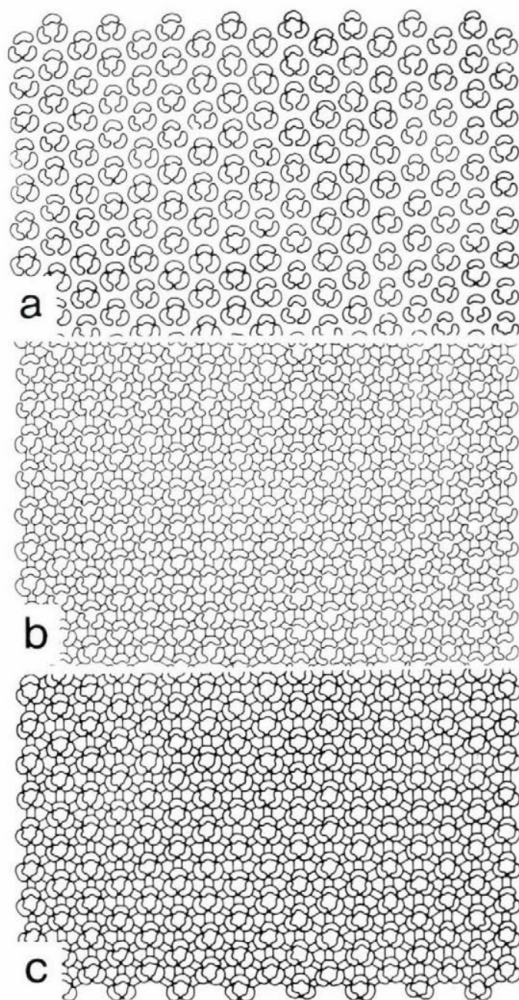


Fig. 10. Tentative diagram of the respective positions of the three polypeptides within a hexon and the molecular arrangement of the inter- and intrahexonal connections. (Explanation in the text)

corresponds to the corner to edge conjunction demonstrated for nonamers in the capsid of the virus [16, 19–21]. This arrangement seems to be the rule for two-dimensional crystalline arrays. Naturally, there might be irregularities in the orientation of the hexons since some rotate a few degrees as compared to the position of the rest. This rotation has its maximum at 60° because of the threefold symmetry. This 60° rotation necessarily modifies the character of the linkages among the hexons.

Part *b* of the diagram represents the position of interhexonal connections. It shows that, on account of the threefold symmetry, both ends of any poly-

peptide of any central hexon are linked with parallel bridges to one end of the two nearest polypeptides of the next hexon. Thus, every hexon is connected to its six nearest neighbours by two approximately parallel connections, i.e. by twelve connections in a regular case. Every parallel pair of connective elements encloses an approximately 60° angle with the neighbouring parallel connections. This leads to the configuration in which the space among the nearest three hexons is divided into four spaces by the interhexonal connections and where the central area has an approximately triangular shape (Fig. 3).

The threefold symmetry allows to assume that a central hexon must possess three times two connections as its "own". These lead from it to three out of its six nearest neighbours, while the "own" connections of the other three neighbouring hexons lead to the central hexon. Four interhexonal connections belong to each polypeptide of a hexon with only two of them being its own; either the two outer connections, which enclose an angle of approximately 120° with each other, or the two inner ones which run parallel.

It is also possible that every hexon polypeptide has four own linkages or projections which link to one another and form the interhexonal connections or a low molecular weight extra polypeptide might step between the corresponding linkage points of two neighbouring hexons.

Considering the fact that the observed connections have stayed with the hexons in spite of the lengthy processes of extraction from infected cells, purification and concentration, it is concluded that they are integral parts of the hexon polypeptides or their synthesis runs parallel to that of the polypeptides and they develop strong links.

Part *c* of the diagram shows the intrahexonal connections. They are marked by fine double lines that may signify both single or double links. On the electron micrographs we generally observe single intrahexonal connections. Still, intrahexonal connections being stronger than interhexonal ones, we might count with double connections as well. It cannot, however, be excluded that these lie not in the plane of the electronmicrographs or drawings but are re-arranged in depth, one below the other, among the polypeptides.

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IgG-Fc-BINDING RECEPTORS IN CELLS ABORTIVELY INFECTED, OR TRANSFORMED, BY HUMAN CYTOMEGALOVIRUS*

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Abortive human cytomegalovirus (HCMV) infection of normal hamster cells is accompanied by formation of cytoplasmic and surface receptors for the Fc portion of nonimmune IgG molecules. The process requires RNA and protein synthesis but no DNA synthesis. The surface receptors are detectable only transiently, in a small proportion of the cells. The receptors are also present in cells transformed by HCMV *in vitro* (87-TRH-5 and CX-90-3B cell lines) as well as in cells of lines TSC-1 and TSC-2 from tumours induced by these cells. Consequently, the indirect immunofluorescence test performed with sera containing no HCMV antibodies may give a positive reaction in the cytoplasm and/or cell membrane of the transformed cells.

Herpes simplex virus infection results in the appearance of IgG-Fc-binding receptors on the cell membrane [1, 2] while in the HCMV-infected cells similar receptors appear in the cytoplasm [3–5]. We have sought IgG-Fc-binding cytoplasmic and membrane receptors in cells abortively infected or transformed by HCMV. The results are reported in this paper.

Materials and methods

Cell cultures. Human embryonic fibroblast (HuEFb) and hamster embryonic fibroblast (HEFb) cultures were prepared from human abortion material and Syrian golden hamster embryos, respectively. The cells had undergone 2–10 passages before use in the experiments. Minimal Essential Medium (MEM) containing 10% heat-inactivated neonatal calf serum, 100 U/ml penicillin, 100 µg/ml streptomycin and 0.076% NaHCO₃ was used as growth and maintenance medium.

The HCMV-transformed cell lines used were: 87-TRH-5 [6], *in vitro* passage No. 98–142; CX-90-3B [7], *in vitro* passage No. 65–105 in our laboratory; TSC-1, a cell line established from the tumour induced with 87-TRH-5 cells in hamsters [6], *in vitro* passage No. 32–65; TSC-2, a cell line established from the tumour induced with TSC-1 cells, *in vitro* passage No. 8–28. The composition of the medium was the same as of the medium used for the HuEFb cells.

Virus. The human cytomegalovirus strain Ad-169 had been kindly supplied by Dr. H. K. ANDERSEN (Institute of Medical Microbiology, University of Aarhus, Denmark). The virus was maintained and its infectivity was titrated as described elsewhere [8].

Indirect immunofluorescence (IF). The method was performed as published elsewhere [9].

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Haemadsorption. Coverslip cultures were rinsed in PBS and overlaid with 100-fold diluted haemolysin. Control cultures were overlaid with 100-fold diluted normal rabbit serum. The preparations were thus incubated at 37 °C for 1 h. After rinsing in PBS for 15 min the cultures were covered with a 0.5% sheep red cell (SRBC) suspension. After another incubation at 37 °C for 1 h, the preparations were washed in PBS for 1 h, then the cells were fixed in 4% formalin and stained in Giemsa solution. Each cell to which at least three SRBCs were attached at the time of reading, was regarded as positive.

HCMV-negative sera used in the IF test. Human sera that had proved negative in the ACIF test [10] or in the CF test [11] as well as serum samples from non-immunized, apparently healthy rabbits and mice were used. The sera were diluted tenfold before use.

Globulin fractions used in the IF test. The human IgG fractions and the rabbit IgG, IgG-Fc and IgG-Fab fractions were prepared [12] and kindly supplied by Dr. S. YUSUPOVA (Institute of Pathological Physiology, University Medical School, Debrecen).

Conjugates. Anti-human IgG, anti-human IgM, anti-human IgA, anti-rabbit IgG and anti-mouse IgG conjugates (Hyland, Division Travenol Laboratories Inc., Costa Mesa, Calif., USA).

Haemolysin. Institute for Serobacteriological Production and Research Humán, Budapest.

Chemicals. Cytosine arabinoside hydrochloride (ara-C) and puromycin hydrochloride (Sigma Chemical Company, St. Louis, Mo., USA). Actinomycin D (Calbiochem, Los Angeles, Calif., USA).

Results

IgG-binding cytoplasmic receptors in HuEFb and HEFb cells infected by HCMV. Cell cultures abortively infected by HCMV were tested for IgG-Fc-binding receptors, and it was studied whether the formation of these receptors requires *de novo* synthesis of DNA, RNA and protein.

In hamster cells infected by HCMV, infectious virus is not produced, though some virus markers including CPE and early antigens, do appear [13]. We have observed cytoplasmic IF in infected hamster cells even with sera negative for anti-HCMV. The cytoplasmic fluorescence was diffuse, and dense bodies often appeared in the cytoplasm of certain cells. The kinetics of formation of the non-specific staining was comparatively followed up in human and hamster cell cultures.

HuEFb and HEFb cell cultures were inoculated with HCMV; the multiplicity of infection (m.o.i.) was 1 or 5 TCID₅₀ virus/cell. The cytoplasmic fluorescence demonstrable with negative sera was followed by the indirect IF test. In these studies anti-HCMV-negative human sera were used with anti-human IgG conjugate. The temporal appearance of the fluorescence independent of the presence of HCMV antibodies was the same in the human and the hamster cells. This type of fluorescence appeared even at 1 m.o.i., but the rate of infected cells was higher if the m.o.i. was 5 TCID₅₀/cell. In the latter case, 5–10% and nearly 100% of the cells, respectively, showed fluorescence with negative sera. In the human cells, cytoplasmic dense bodies were seen, whereas in the hamster cells the fluorescence was generally diffuse, with inclusion-like bodies in 20–30% of the cells.

Figure 1 shows human cells tested with serum containing no anti-HCMV antibody in the 72nd h after inoculation.



Fig. 1. Inclusion-like body in the cytoplasm detectable with HCMV negative serum; 72 h postinfection

Next, the non-HCMV-specific fluorescence was examined with negative human sera and anti-human IgM and IgA conjugates as well as with sera or serum fractions from nonhuman species and the corresponding conjugates.

HuEFb and HEFb cells were fixed 72 h after an inoculation at 5 m.o.i. and treated with sera or serum fractions obtained from different nonhuman species and with the corresponding conjugate. The results presented in Table I show that no fluorescence appeared in the presence of anti-human IgM or IgA conjugates and, among the anti-HCMV-negative globulin fractions of rabbit sera the IgG-Fab fractions did not bind to the cells. If whole rabbit serum, or its IgG or IgG-Fc fraction, was used with anti-IgG conjugate, cytoplasmic fluorescence appeared in both human and hamster cells, suggesting that IgG-Fc-binding receptors had formed in the cytoplasm of both the hamster and the human cells and that these receptors are responsible for the fluorescence appearing with anti-HCMV-negative sera.

No fluorescence was observed in non-infected human and hamster cells.

To examine the inhibition of macromolecule synthesis, we added inhibitors to infected cultures 1 h after the inoculation and fixed the cultures after a further incubation of 72 h. The m.o.i. was 5 TCID₅₀/cell. HCMV-negative rabbit serum was used with anti-rabbit IgG conjugate (Table II). The development of cytoplasmic fluorescence was inhibited by inhibitors of RNA synthesis and by those of protein synthesis. In the presence of ara-C, the infected HuEFb cultures did not develop cytoplasmic inclusion-like bodies while the diffuse fluorescence and in a few cells inclusions, did appear in the cytoplasm of HEFb. Cytoplasmic fluorescence occurred in the HCMV-infected hamster cell cultures not treated with ara-C less frequently (45%) than in the ara-C-treated similar cultures (65%).

Table I
*Cytoplasmic diffuse fluorescence and dense bodies in HuEFb and HEFb cells infected by HCMV**

Treatment		HuEFb		HEFb	
HCMV-negative serum or serum fraction	Conjugate	Cytoplasmic dense bodies	Nucleus	Cytoplasmic diffuse IF and dense bodies	Nucleus
Human adult serum n=30	IgG	80-100	—	80-100	—
	IgM	—	—	—	—
	IgA	—	—	—	—
Infant serum n=20	IgG	80-100	—	80-100	—
	IgM	—	—	—	—
	IgA	—	—	—	—
Rabbit serum n=7	IgG	80-100	—	80-100	—
Rabbit IgG		80-100	—	80-100	—
Rabbit IgG-Fc		80-100	—	80-100	—
Rabbit IgG-Fab		2-3	—	2-3	—
Mouse serum n=20	IgG	80-100	—	80-100	—

* Positive cells per cent, determined with indirect IF

Table II
*Influence of inhibitors of macromolecule synthesis on the formation of cytoplasmic IgG-Fc-binding receptors**

Inhibitor		HuEFb		HEFb	
Designation	Concentration, $\mu\text{g/ml}$	Cytoplasmic dense bodies	Nucleus	Cytoplasmic diffuse IF and dense bodies	Nucleus
No inhibitor	—	96	—	45	—
Ara-C	15	2	—	65	—
Puromycin	50	—	—	—	—
Actinomycin D	1	—	—	—	—

* Positive cells, per cent, determined with indirect IF

We failed to detect cytoplasmic fluorescence in cells not inoculated with HCMV.

IgG-Fc-binding receptors on the cell membranes of HuEFb and HEFb cells infected by HCMV. Human and hamster embryonic fibroblasts were

inoculated with the m.o.i. 1 or 5 TCID₅₀ virus/cell. The living cells were examined with the IF and the haemadsorption method. In the IF test, HCMV-negative human and rabbit sera were used. In the cultures inoculated with 1 TCID₅₀/cell, no IgG-Fc-binding membrane receptors could be detected at any time after inoculation. Table III shows the results obtained by inoculation with 5 TCID₅₀/cell. The results showed that the IgG-Fc-binding receptors required a m.o.i. not less than 5 TCID₅₀ virus/cell to be expressed in a low percentage (12–15%) of cells, and that the expression was transient. The results of the IF test and those of the haemadsorption test correlated well. With the non-infected cells neither IF nor haemadsorption could be observed.

Table III

*Formation of IgG-Fc-binding receptors on the surface of HuEFb and HEFb cells infected by HCMV**

Cells	Time after virus inoculation, h									
	48	72	96	120	144	48	72	96	120	144
	immunofluorescence					haemadsorption				
HuEFb	1	7	1	1	n.t.	2	9	3	2	n.t.
HEFb	1	10	12	6	1	3	15	11	3	2

* Positive cell, per cent

The results of treatments with inhibitors of macromolecule synthesis, viz. actinomycin D and ara-C, are shown in Table IV. Puromycin was not examined because, in the applied concentration (50 µg/ml), this drug was injurious for the cells. The formation of IgG-Fc-binding receptors was pre-

Table IV

*Influence of inhibitors of macromolecule synthesis on the formation of IgG-Fc-binding receptors in the cytoplasmic membrane**

Inhibitor		HuEFb cells		HEFb cells	
Designation	Concentration, µg/ml	IF	Hads**	IF	Hads
No inhibitor	—	6	11	11	14
Ara-C	15	4	8	73	85
Actinomycin D	1	—	—	—	—

* Positive cells, per cent

** Haemadsorption

vented by actinomycin D, but was not affected by ara-C. Interestingly, the proportion of hamster cells bearing IgG-Fc-binding receptors was substantially increased in the presence of ara-C.

IgG-Fc-binding receptors in the cytoplasm and on the cytoplasmic membrane of HCMV-transformed cells. 87-TRH-5, CX-90-3B, TSC-1 and TSC-2 cells were cultivated on coverslips for 2 days. The monolayers were examined for IF in living and fixed states and for haemadsorption in the living state.

HCMV-negative human sera, human IgG fraction, rabbit sera, the IgG, IgG-Fc and IgG-Fab fractions of rabbit sera, and mouse sera were tested for IF. The results are presented in Table V. The negative complete sera and the fractions IgG and IgG-Fc were found to be bound to the cells; these were either fixed or non-fixed. The IgG-Fab fraction was not bound. In the fixed preparations, diffuse fluorescence and at places inclusion-like bodies were detected in the cytoplasm. The fixation of rabbit sera and of its fractions to the IgG-Fc-binding surface receptors of TSC-2 cells was more pronounced than the fixation of human sera, human IgG or mouse sera.

Table V

*IgG-Fc-binding activity in the cytoplasm and in the membrane of cells transformed by HCMV**

HCMV-negative serum or serum fraction	Cells							
	87-TRH-5		CX-90-3B		TSC-1		TSC-2	
	CP	CM	CP	CM	CP	CM	CP	CM
Human serum	92	95	94	95	85	83	83	12
Human IgG	98	94	93	93	84	82	98	13
Rabbit serum	98	97	95	93	89	86	81	76
Rabbit IgG	96	92	96	92	87	82	84	78
Rabbit IgG-Fc	95	97	93	95	85	81	82	76
Rabbit IgG-Fab	5	5	4	3	6	5	5	4
Mouse serum	92	87	92	93	82	76	87	14

* Detected by indirect immunofluorescence using IgG conjugate, per cent; CP = cytoplasmic IF; CM = membrane IF

The results of the haemadsorption tests are shown in Table VI. Most haemolysin-treated cells formed rosettes with SRBC. Among the cells pre-treated with rabbit serum or PBS, the 87-TRH-5 cells did, the other cells did not, form rosettes with SRBC. Still, like other cells, the 87-TRH-5 cells did not form rosettes with chicken erythrocytes without specific treatment. The cause of spontaneous rosette formation with SRBC is to be clarified.

Table VI*IgG-Fc-binding activity on the cytoplasmic membrane of cells transformed by HCMV**

Treatment of cells	Cells			
	87-TRH-5	CX-90-3B	TSC-1	TSC-2
Normal rabbit serum +SRBC	90	5	3	2
Haemolysin+SRBC	98	89	78	72
PBS+SRBC	91	2	2	2
PBS+chicken erythro- cytes	2	1	1	1

* Positive cells, per cent, determined with haemadsorption

Discussion

The presence of IgG-Fc-binding receptors in cells productively infected by HCMV and several properties of these receptors have been described [3-5, 14]. It is well known, e.g., that the receptors are glycoproteins of 4.2×10^4 dalton mol wt; they are continuously produced after an early period of infection [5]. Dense bodies bearing IgG-Fc receptors have been isolated and were found to contain a great part of the structural proteins of the HCMV virion, but no DNA [14]. The receptors were detected also on the surface of cells infected by HCMV [4]. It was, however, not clear which of the inhibitors of macromolecule synthesis inhibit the synthesis of the receptors, and whether they are formed during abortive virus infection and in HCMV-transformed cells.

In the present work, we have shown that cytoplasmic receptors are formed in abortively infected cells, too (Table I), and that similar receptors are formed on the cell surface of a low proportion (12-15%) of the cells infected either productively (human cells) or abortively (hamster cells); see Table III. The formation of these receptors requires *de novo* RNA and protein synthesis; if, however, DNA synthesis is inhibited with ara-C, the receptors are formed in both the cytoplasm and the cell membrane of the abortively infected cells more readily than without ara-C (Table II and IV). This might be related to the observation that non-permissive cells may turn into permissive cells (i.e. cells shedding virus) [15] or to an observation in another system, viz. that the number of cells containing virus-specific antigen can substantially be raised by inhibiting their DNA synthesis with IUdR [13].

Surprisingly, cytoplasmic IgG-Fc receptors had formed in a very low proportion (2%) of the human cells and in a much higher proportion (65%) of the hamster cells while DNA synthesis was inhibited. Presumably, the

mechanisms contributing to the formation of receptors in permissive cells are different from the mechanisms functioning in non-permissive cells.

Surface receptors binding to the Fc fragments of normal immunoglobulins have been described on lymphoid, reticulo-endothelial and tumour cells [16, 17]. We cannot exclude the possibility that the IgG-Fc-binding receptors found on the surface of the transformed cells are unrelated to the fact that the transformation had been induced with CMV. The cytoplasmic dense bodies in the transformed cells are, however, morphologically so similar to the inclusion-like bodies in the HCMV-infected cells that we cannot dismiss the thought that the dense bodies may be products of the HCMV genome.

IgG-Fc-binding receptors are transiently present in the cytoplasm and on the surface of cells being abortively infected by HCMV; HCMV-specific antigens are also formed transiently [13, 18]. It might be assumed that during HCMV infection *in vivo*, while IgG-Fc-binding receptors are present, cytolysis is prevented owing to the presence of the receptors and the consequent fixation of IgG molecules; later on, when the antigens are not expressed, the cells become unrecognizable for the immune system.

The transformed cells are very rich in IgG receptors. According to ADLER *et al.* [18], the immune complexes bound to the receptors cover the tumour-specific antigen and thus prevent the specific antibodies and/or cytotoxic immune cells from being bound to the tumour cells. This hypothesis includes the assumption that the IgG-Fc-binding receptors and the tumour specific antigens are located near to each other or that the IgG-Fc fraction binds to the tumour-specific antigens.

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GUANOSINE POLYPHOSPHATE PRODUCTION OF *ESCHERICHIA COLI* STRINGENT AND RELAXED STRAINS IN THE STATIONARY PHASE OF GROWTH

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Stringent and relaxed *Escherichia coli* strains grown on minimal and on differently enriched media produced guanosine polyphosphates in the stationary phase of growth. On transition from the logarithmic to the stationary phase, stringent strains started to produce guanosine 5'-triphosphate 3'-diphosphate (pppGpp), and guanosine 5'-diphosphate 3'-diphosphate (ppGpp), while relaxed strains accumulated only ppGpp. When the stringent strain was cultivated on media enriched with Casamino Acids the level of pppGpp decreased, while with yeast extract an almost twofold increase could be observed. The concentration of ppGpp increased with both nutrients as compared to that measured in minimal medium. Readdition of glucose to the stationary phase culture did not result in the slightest decrease of the nucleoside polyphosphate levels. In contrast, addition of a mixture of 20 L-amino acids or Casamino Acids or yeast extract to the medium caused an abrupt decrease in the guanosine polyphosphate levels. Qualitatively similar results were obtained with the relaxed strains except that the amounts of ppGpp were smaller than in the case of the stringent counterpart and the responses to the resupplementation were slower. Some possible mechanisms regarding the occurrence of guanosine polyphosphates in the stationary phase are discussed.

During the past few years a great deal of knowledge has been accumulated concerning the mechanism of growth rate transitions induced in the logarithmic phase by amino acid or carbon source starvation [1, 2]. The regulatory role of ppGpp in these processes has been substantiated by experimental data. Recently, several groups have elucidated the complex relationship between polyposphorylated guanine nucleotides, called the ppGpp cycle [3–5].

Certainly, a much more complex metabolic change takes place when from balanced growth, microorganisms turn to the stationary phase. Regulatory patterns of growth transition have not, however, been studied in detail.

For *Bacillus subtilis*, RHAESE *et al.* [6] observed the appearance of some six highly phosphorylated nucleotides, with pppGpp and ppGpp among them at the beginning of the sporulation. Still, these two nucleotides do not seem to exert any important regulatory function in the course of differentiation [7].

In *E. coli* strains the onset of the stationary phase is accompanied by a drastic reduction in the rate of macromolecule synthesis. More recently, KAWAKAMI *et al.* [8] showed that these changes originated partly from the reduced number of functionally active RNA polymerase molecules and partly from the partial repression of the *E. coli* genome.

The macromolecular pattern of the bacterial cell in the logarithmic phase can also be affected by the composition of the medium (minimal or enriched) by the adjustment of the amounts of total proteins [9], as well as the distribution of ribosomal proteins [10, 11]. It seems therefore that a somewhat different kind of regulation of macromolecule synthesis may be effective during the stationary phase of *E. coli* grown on rich compared to minimal medium. This has prompted us to investigate how the level of the regulatory nucleotides pppGpp and ppGpp change in the stationary phase of *E. coli* K12 stringent and relaxed strains, grown on enriched media.

Materials and methods

Strains and growth conditions. The *E. coli* K12 strains used were, CP 78 (N. Fiil strain CGSC: 4695, *rel* A⁺) and its relaxed counterpart, CP 79 (N. Fiil strain CGSC: 4696, *rel* A). Strains were grown under shaking at 37 °C on Tris-minimal medium [12] supplemented with the required amino acids (50 µg/ml each, except serine which was used at 200 µg/ml concentration), with thiamine (60 µg/ml) and with glucose (0.4% w/v) as carbon source. Phosphate concentration of the medium was 1 µmole/ml. Growth was monitored turbidimetrically at 550 nm (A_{550}).

Assay of pppGpp and ppGpp levels. From a subculture grown on Tris-minimal medium overnight, a small inoculum was transferred into a new medium enriched with the appropriate nutrients. At $A_{550} = 0.150$ the culture was labelled with ³²P-phosphate (carrier free) to a specific activity of about 200 µCi/µmole. After one generation time, to aliquots of the culture the following nutrients were added: (a) mixture of 20 L-amino acids (final concentration 50 µg/ml each); (b) Casamino Acids (Difco), 0.2% w/v; and (c) yeast extract (Difco), 0.2% w/v. From the beginning of the late logarithmic phase 50 µl samples were withdrawn and pipetted into 5 µl 4 M formic acid. The procedure applied thereafter was essentially the one by CASHEL [13]. Nucleotides were separated on PEI cellulose thin layer sheets (Macherey-Nagel), and developed in 1.5 M KH₂PO₄, pH 3.4 and autoradiographed. Spots corresponding to (p)ppGpp were cut out and counted in water by the Cerenkov effect, using a Beckman Liquid Scintillation Spectrometer Model LS-355. Results were expressed as nmole nucleotide/ml A_{550} .

Resupplementation with various nutrients. The culture grown on 0.2% (w/v) yeast extract or on 0.2% (w/v) Casamino Acids was allowed to reach the stationary phase. After about 120 min from the onset of the stationary phase, the medium was resupplemented with glucose (up to 0.4% w/v), yeast extract (up to 0.2% w/v), or with the mixture of the 20 L-amino acids (100 µg/ml each). Changes in the nucleoside polyphosphate levels and in the A_{550} of the culture were followed by sampling at appropriate times as described above.

Identification of the nucleoside polyphosphates. This was carried out according to established procedures [14, 15]. It involved digestion of the prepared and partially purified [14] compounds with HCl and KOH, used in both dilute and concentrated form, splitting with inorganic pyrophosphatase, followed by a treatment with 3' nucleotidase and assay of the various degradation products by two dimensional PEI thin layer chromatography in various solvent systems [14]. These experiments verified our assumption that the nucleotides were pppGpp and ppGpp.

Results

During their transition from the logarithmic to the stationary phase, both the stringent (CP 78) and the relaxed (CP 79) strains accumulated guanosine polyphosphates (Fig. 1). In the stringent strain pppGpp and ppGpp, in the relaxed mutant only ppGpp was synthesized.

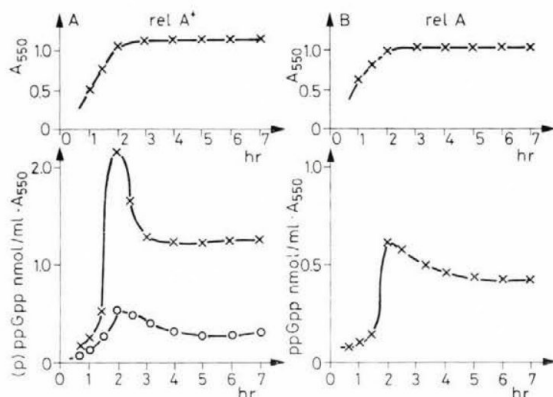


Fig. 1. Guanosine polyphosphate production in the stationary phase of the stringent CP 78 (A) and the relaxed CP 79 (B) strains in minimal medium. $\circ-\circ$ pppGpp; $\times-\times$, ppGpp. On the upper part of the figure the growth curve (A_{550} vs time) is shown. During the transition phase of growth, sampling was done at 2–5 min intervals. For simplicity, however, several intermittent values have been omitted from the figure. Each of our figures display the results of a representative experiment. In all the figures, nucleoside polyphosphate concentrations are calculated by the method originally described by LAZZARINI and CASHEL [20]

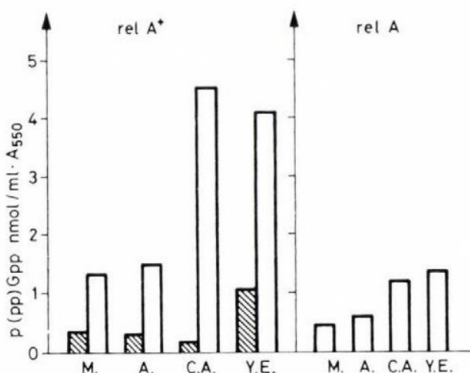


Fig. 2. Effect of various nutrients on the guanosine polyphosphate production of stringent CP 78 (left panel) and relaxed CP 79 (right panel) strains. Bacteria were grown in the presence of the following nutrients: minimal medium (M); a mixture of twenty L-amino acids, 50 $\mu\text{g}/\text{ml}$ each (A); Casamino Acids 0.2% (C.A.); yeast extract, 0.2% (Y.E.), final concentrations. Columns represent steady state values measured approximately two hours after onset of the stationary phase. Dashed columns: pppGpp; empty columns: ppGpp

When the stringent strain was grown in Tris-minimal medium the levels of pppGpp and ppGpp were similar to those measured during amino acid starvation in the logarithmic phase.

In the stringent strain enrichment of the medium with either yeast extract or Casamino Acids elicited a substantial rise in the ppGpp level, while growth on minimal medium plus amino acid mixture resulted in a small

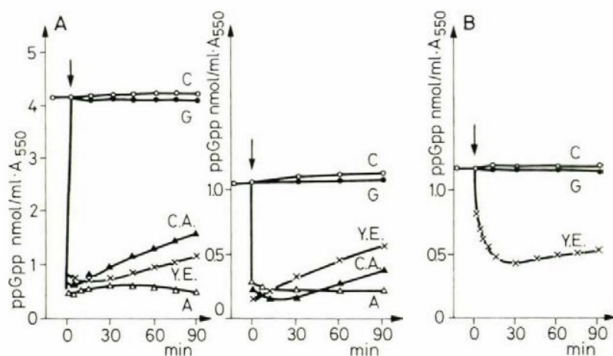


Fig. 3. Effect of readdition of various nutrients to the stationary phase cultures of stringent and relaxed strains. Part A: CP 78, ppGpp (left) and pppGpp (right). Part B: CP 79, ppGpp. Arrows indicate the time of replenishment (zero time). Abbreviations are the same as in Fig. 2. G: 0.4% glucose; C: control. Amino acid mixture was added at 100 $\mu\text{g/ml}$ final concentration

increase (Fig. 2, left panel). ppGpp/pppGpp ratios were close to 4 in every case except with Casamino Acids where it exceeded 20.

For the relaxed strain, enrichment of the minimal medium with Casamino Acids or yeast extract gave rise to a marked increase of the ppGpp level (Fig. 2, right panel), while pppGpp could not be detected.

The slowing down of balanced growth, as well as the elevated synthesis of guanosine tetraphosphate in different media might be attributed to different reasons (as amino acid or carbon source starvation, etc.). To select among the possibilities, supplementation of the guanosine polyphosphate producing culture was performed with the appropriate nutrient. From Fig. 3 it is apparent that for the stringent strain addition of amino acid mixture or Casamino Acids or yeast extract to the culture drastically suppressed both pppGpp and ppGpp levels when either Casamino Acids or yeast extract were used in the original medium. (In Fig. 3 experiments with a medium containing yeast extract are presented.) Decrease of the guanosine polyphosphate level was accompanied by an increase in the growth rate.

Addition of glucose to the stationary phase guanosine polyphosphate producing stringent strain did not cause any notable effect in the (p)ppGpp levels.

In the relaxed strain, grown on yeast extract, the elevated ppGpp level could be suppressed by yeast extract only. The time course of the decrease was, however, much slower than that with the stringent strain. Glucose did not affect either ppGpp level or the growth rate.

All the above experiments were repeated with another stringent-relaxed pair, CP 99 and CP 100, and similar results were obtained (data not shown).

Discussion

The fact that on transition to stationary phase of growth, both the stringent and the relaxed strains of *E. coli* produced (p)ppGpp, was not surprising *per se* since guanosine polyphosphate synthesis went parallel with the slowing down of the growth. It is interesting, however, that the level of guanosine tetraphosphate in either strain was considerably higher with enriched media than with the Tris-minimal one.

In the stringent strain, the highly increased pppGpp level was probably induced by an enhanced amino acid starvation. This assumption is supported by the results of resupplementation experiments which in turn exclude the direct involvement of glucose starvation.

The highly elevated ppGpp levels and particularly the great difference in the ppGpp/pppGpp ratio in the stringent strain using Casamino Acids or yeast extract in the growth medium are puzzling. Although it cannot be interpreted satisfactorily on the basis of the available experimental data, some assumptions might be risked. It is less probable that the high level of ppGpp would be a consequence of the impaired ppGpp degradation [16-18], since the readdition of amino acids mixture caused a very rapid decay. Readdition of some components other than amino acids (nucleotides, vitamins) of the semisynthetic media did not cause any appreciable change in the level of guanosine polyphosphates (data not shown). It might be inferred that in the two nutrients the contribution of the *rel A* dependent (stringent factor catalysed) [1] and *rel A* independent [2, 19] synthesis routes to the ppGpp level is different. In the case of Casamino Acids the activity (or the amount) of stringent factor would be decreased (low pppGpp level), while with yeast extract an activation of the *rel A* gene product might occur.

In the relaxed mutant the occurrence of a simple glucose starvation can be excluded. The increased synthesis of ppGpp might be attributed, in part, to the activation of *rel A* independent reaction(s) and, in part, to the inhibition of ppGpp degradation [16-18]. The latter mechanism is substantiated by the relatively slow decay of ppGpp on readdition of yeast extract to the stationary phased culture.

In conclusion we have to emphasize that although we interpreted our results in terms of the starvation in the logarithmic phase, these are not necessarily valid in each detail in the stationary phase where much more complex relationships may exist.

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KLEBSIELLA AND ENTEROBACTER STRAINS DERIVED FROM HOSPITAL INFECTIONS

II. OCCURRENCE AND CHARACTERIZATION OF R-, LAC- AND COL-PLASMIDS AND THEIR CLINICAL-EPIDEMIOLOGICAL SIGNIFICANCE

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A total of 269 hospital *Klebsiella* strains and 103 hospital *Enterobacter* strains showed 34 and 10 different antibiotic resistance patterns, respectively. Among multiple resistant *Klebsiella* and *Enterobacter* strains the Ap Sm Cm Tc resistance pattern was the most frequent (*K. aerogenes*). Antibiotic resistant strains carried R-plasmids in 27.5%. The presence of R-plasmids was demonstrable in 2.9% of single antibiotic resistant, in 12.8% of double antibiotic resistant, and in 71.4% of multiple antibiotic resistant *Klebsiella* strains. R-plasmid carriage was most frequent in strains of the species *K. pneumoniae* and *K. atlantae*. Transfer frequency of R-plasmids of multiple resistant strains was generally higher into *Escherichia coli* K12 recipient (10^{-1} to 10^{-2}) than into *K. pneumoniae* recipient (10^{-3} to 10^{-5}). Regarding the individual resistance determinants, transfer frequency of Km-Nm resistance was the highest (65.1%). The Lac-plasmid was demonstrable in 18 out of the 77 R-plasmid carrying *Klebsiella* strains. R- and Lac-plasmids of single and multiple resistant *Klebsiella* strains could be transferred into *E. coli* and *Klebsiella* recipients in mice *in vivo*. As many as 67% showed *fi*⁻ and 33% showed *fi*⁺ character among the demonstrated 112 R-plasmids. Regarding their incompatibility, the studied five plasmids belonged to groups FI, FII and Iz. Altogether 14 different groups could be distinguished among R-plasmids on the basis of their phage restriction capability on phage λ and coliphages T, and another group of R-plasmids showing no restriction at all. *Fi*⁺ character was demonstrated in 54.5% of R-plasmids showing phage restriction capability and in 10.9% of R-plasmids showing no phage restriction. Nearly twice as many R-plasmid carrier strains were found among non-typable ones as among those typable by phages. Three R-plasmids showed phage-modification among 51 R-plasmids restrictive for phages. In addition to the phage-type of *Klebsiella* strains, R-plasmids can also be used as an epidemiologic marker.

In an earlier paper [1] data have been reported on the classification into species of *Klebsiella* and *Enterobacter* strains of hospital origin, their subdivision by phage-typing and on their antibiotic resistance. In the course of the present study we examined the frequency of antibiotic resistance determining plasmids in *Klebsiella* and *Enterobacter* strains. The R-plasmid carrier state of the two genera, the influence of the plasmids on the phage-type and their phage restriction capacity were studied. The study on R-plasmids was extended to observations on the presence of Lac- and Col-plasmids as well. Transfer frequency of R- and Lac-plasmids was studied *in vivo*.

Materials and methods

Bacterial strains examined for antibiotic resistance and R-plasmid carriage. A total of 470 strains belonging to genus *Klebsiella*, and 103 strains belonging to genus *Enterobacter* were studied. The origin of these strains was described previously [1].

Donor strains. A total of 269 single and multiple resistant *Klebsiella* strains, and 6 single and multiple resistant *Enterobacter* strains were examined for R- and Lac-plasmid carriage.

Recipient strains. In the course of R-, Lac- and Col-plasmid transfer experiments the strains used as recipients were: *E. coli* K12 HfrH met⁻ lac⁻ nal^r, *E. coli* K12 C600 F⁻ thr⁻ leu⁻ thi⁻ lac⁻ nal^r, *E. coli* J53 R⁻ lac⁺ nal^r, *E. coli* J53 R⁻ lac⁺ sm^r, and *K. pneumoniae* 43. The last strain was isolated in our laboratory, and belonged to phage type XA1; it was sensitive to every antibiotic examined and a nalidixic acid resistant mutant was used as recipient in the transfer experiments.

Phage propagating strains. For propagation of coli phage T *E. coli* B, for phage λ vir *E. coli* C600 F⁻, for phage P1kc *E. coli* J53 and J53 (RN3), for phage MS2 *E. coli* C3000, for phage f2 *E. coli* 61 Hfr, and for phage Ike *E. coli* 264 A strains were used.

Indicator strains for detection of colicin production. Strains *E. coli* K12, *E. coli* Phi (MacGeachie) and *S. sonnei* 16 (Abbott-Shannon) were used.

Reference plasmids. Plasmids 51 (pIP112), 11 (Y. A. CHABBERT, Paris), 55 (pIP175), 12 (Y. A. CHABBERT, Paris), 62 (RP4), P (Y. A. CHABBERT, Paris), 64 (pIP517), C (Y. A. CHABBERT, Paris), 72 (pIP72), 10.B.O. (Y. A. CHABBERT, Paris), 73 (TP116), H (Y. A. CHABBERT, Paris), 67 (pIP518), M (Y. A. CHABBERT, Paris), J53 (RN3), N (N. DATTA, London), J53 (S-a), W (N. DATTA, London) and J53 (R64), Iz (N. DATTA, London) were used as reference plasmids in the course of the experiments.

Phages used for phage-typing have been described earlier [1].

Phages used for determination of *fi* character. Determination of *fi* character was carried out using male specific RNA phages MS2 [2] and f2 [3].

Phages used for phage restriction and phage modification experiments. Phage λ vir, coliphages from T1 to T7, phage P1kc [4] and phage Ike [5] were used.

Media. Media used for propagation of phages and for phage-typing. Beef extract agar and soft agar were used. The soft agar was the same as the former except that it contained a half amount of agar.

Media used in R-, Col- and Lac-plasmid transfer experiments. Eosin methylene blue agar and beef extract agar were used. The concentration of antibiotics in the media during *in vitro* transfer experiments were, chloramphenicol, tetracycline and kanamycin, 20 μ g/ml; streptomycin and ampicillin, 30 μ g/ml; and nalidixic acid, 50 μ g/ml. A concentration of 50 μ g/ml of ampicillin was used instead of 30 μ g/ml in experiments where the *Klebsiella* strain was used as recipient.

In experiments *in vivo*, the following concentrations of antibiotics were used: streptomycin 25 μ g/ml, chloramphenicol 10 μ g/ml, tetracycline 12.5 μ g/ml, neomycin and ampicillin 20 μ g/ml, and nalidixic acid 50 μ g/ml.

Preparation of the above-mentioned media was carried out according to the Guidelines of the National Institute of Hygiene, Hungary [6].

Antibiotic sensitivity tests were carried out on modified Mueller-Hinton agar [7].

In MIC value determinations, N5 Difco pH 8.0 medium was used, supplemented with the appropriate antibiotics [8].

In the course of fertility inhibition experiments, a minimal medium was used [9] supplemented with 2.5 μ g/ml of thiamine [10]. Minimal medium supplemented with 50 μ g/ml of trimethoprim (Tp) and 50 μ g/ml of methionine was used in controlling trimethoprim resistance transfer [11].

Agar diffusion antibiotic sensitivity tests. The following "Resistest" (Institute for Sero-bacteriological Production and Research Humán, Budapest) disks were used: ampicillin (Ap), streptomycin (Sm), chloramphenicol (Cm), tetracycline (Tc), kanamycin (Km), neomycin (Nm), polymyxin-B (Pm), colistin (Co), gentamicin (Gm) and nalidixic acid (Nal).

Trimethoprim sensitivity tests were carried out in Mueller-Hinton medium using serial twofold dilutions of the drug from 5 to 1000 μ g/ml.

MIC value determination. MIC (Minimum Inhibitory Concentration) determinations were carried out according to the WHO prescription [12].

MIC values of a total of 167 strains were determined of which 30 strains showed single, and 137 multiple, resistance against antibiotics.

R-plasmid transfer experiments *in vitro*. The method of WATANABE and FUKASAWA [13] was used with slight modifications. Donor and recipient strains were preincubated separately at 37 °C for 18 h. A volume of 0.5 ml from these cultures was inoculated into 4.5 ml of fresh broth, and incubated further at 37 °C for 3 h.

Taking a volume of 0.1 ml from the donor culture, and 0.9 ml from the recipient, a mixture was prepared, and incubated at 37 °C for 18 h. For selection, 0.1 ml fractions of the mixture were spread onto selective plates, and the plates were incubated for 48 h. Colonies grown

in the course of the next 24 to 48 h were picked up and subcultured on agar plates containing antibiotics.

Unsuccessful transfer experiments were repeated using the method of LAUTROP *et al.* [14]. Aliquots of 0.1 ml from the 18 h cultures of both the donor and the recipient was pipetted into 60 ml of fresh broth in a 500 ml Erlenmeyer flask and incubated at 37 °C for 42 h. From this mixture 0.1 ml fractions were plated on selective plates.

R-plasmid transfer experiments in vivo. R-plasmid transfer experiments in mice were carried out using the basic method of WATANABE [15] modified by RAUSS and KÉTYI [16]. In each test group 5 mice were included, pretreated with 50 µg/ml streptomycin orally during 2 days. On the 3rd day the enteric bacterial flora was checked on eosin methylene blue agar.

Eighteen hours old agar slope cultures of recipient strains (*E. coli* K12 C600 F⁻, and *K. pneumoniae* 43, an antibiotic sensitive, phage-type XA1 strain) and of donors (*K. aerogenes* K31 resistant to Ap, and K359 resistant to Ap Sm Cm Tc Km Nm) were resuspended in physiological saline. From the recipient suspensions 0.5 ml was given orally to each mice. On the same day the same volume of donor suspension was given orally to mice. Both the recipient and the donor suspensions were prepared in the same way; the number of colony forming units was 3.2×10^7 in each case. After 24 h the faeces of mice were streaked on eosin methylene blue agar plates, and the plates were incubated at 37 °C. From each plate 100 colonies grown until the 24th and the 48th h were transferred on agar plates using tooth-picks, and after incubating for 24 h the colonies were replicated [17] onto eosin methylene blue agar plates containing the mentioned antibiotics. After incubation for another 24 to 48 h the antibiotic-resistant colonies were subcultured on antibiotic containing selective plates. Exconjugants were checked by agar diffusion antibiotic sensitivity test, and phage-typing. The procedure was repeated, but this time before streaking the diluted faeces of mice was treated with donor and recipient bacteria, from 10^{-1} to 10^{-5} in both nalidixic acid free and nalidixic acid containing broth. From each dilution, 0.1 ml portions were plated on antibiotic and nalidixic acid containing eosin methylene blue agar plates and subsequently incubated at 37 °C.

Transfer of Lac-plasmids. (1) In both *in vitro* and *in vivo* systems, the experiments were carried out with R-plasmid transfer experiments using antibiotic containing eosin methylene blue medium for selection. In experiments using *E. coli* lac⁻ recipient lac⁺ transconjugant colonies were sought. (2) The cellophane-membrane method was carried out according to CHABBERT and PATTE [18]. One ml aliquots of both the donor and the recipient cultures were pipetted onto a cellophane membrane placed on agar surface. After incubation at 37 °C for 18 h the membrane was washed with 1 to 2 ml of physiological saline and the suspension was plated on nalidixic acid containing eosin methylene blue agar at a dilution of 10^{-6} . The plates were incubated at 37 °C for 24 to 48 h, lac⁺ colonies were subcultured on nalidixic acid containing eosin methylene blue agar, and then on antibiotic containing agar.

Transfer of Col-plasmids. The cellophane membrane method [18] was used. For selection, nalidixic acid and colicin containing agar plates were used. The colicins used were the filtrates of donor cultures which were plated on nalidixic acid containing agar at a dilution of 1 : 10. In this way, of the recipient cells only the Col-plasmid containing ones were able to grow. In each experiment 50 colonies of those grown in the course of 24 to 48 h incubation were pricked in agar plates. From these plates colonies were replicated onto another agar plate to test their colicin production, and onto agar plates containing antibiotics. The frequency of Col-plasmid transfer was determined as the ratio of the number of exconjugant and of donor colonies.

Examination of colicin production. Examination of colicinogeny were carried out with a modified method of ABBOTT and SHANNON [19].

Determination of transfer frequency of R- and Lac-plasmids. Transfer frequency was calculated by dividing the number of exconjugant colonies by the colony number of the donor strain. In the case of *in vivo* transfer the value was calculated as the average of 5 mouse tests.

Determination of *fi* character of R-plasmids. According to WATANABE *et al.* [20], *fi* character of R-plasmids was determined by testing the phage sensitivity of the recipient *E. coli* K12 Hfr strain against the phages MS2 and f2.

To demonstrate fertility inhibition, cross experiments were carried out with a number of R-plasmids. In these experiments strain *E. coli* HfrH met⁻ carrying the respective R-plasmids was crossed with strain *E. coli* F⁻ thr⁻ leu⁻ ad⁻ his⁻ arg⁻ B1⁻ and the transfer of both markers threonine and leucine were observed using appropriately supplemented minimal medium.

Examination of phage restriction and phage modification. The hsp II phage restriction caused by R-plasmid was determined according to BANNISTER and GLOWER [4]. Phages λ and P1kc were propagated on both strains J53 R⁻ and J53 (RN3) since R-plasmid N3 determines the hsp II phage restriction and modification [21]. Both phages were titrated on the propagat-

ing strains J53 R⁻ and J53 (RN3), as well as on a series of J53 R⁺ strains carrying R-plasmids from different *Klebsiella* and *Enterobacter* strains. The e.o.p. values were determined, and a comparison was made between the e.o.p. values of R⁻ and R⁺ J53 strains. Restriction of phage λ and coliphages T1-T7 was determined in the case of the same plasmids present in *E. coli* K12 HfrH recipient, and e.o.p. values were also determined with this different system.

Examination of incompatibility. These examinations were carried out according to the method of CHABBERT *et al.* [22].

Results

Antibiotic sensitivity of the strains. Data on antibiotic sensitivity of the strains were shown in part (distribution of antibiotic resistance according to species; occurrence of multiple resistance) in an earlier paper [1]. Resistance patterns observed with a frequency of less than 2.0% were pooled in the row "Others". Marking of individual resistance patterns are shown in Table I for *Klebsiella* and in Table II for *Enterobacter* strains.

Table I
Antibiotic resistance patterns of *Klebsiella* strains

Designation of resistance pattern	Ap	Sm	Cm	Tc	Nm	Km	Gm	Pm	Co	NaI	Total	
											No.	%
1	—	—	—	—	—	—	—	—	—	—	28	6.0
2	+	—	—	—	—	—	—	—	—	—	214	45.5
3	+	—	—	+	—	—	—	—	—	—	29	6.2
4	+	+	—	+	—	—	—	—	—	—	20	4.3
5	+	+	+	+	—	—	—	—	—	—	33	7.0
6	+	+	+	+	—	—	—	—	—	+	16	3.4
7	+	+	+	+	+	+	—	—	—	—	31	6.6
8	+	+	+	+	+	+	—	—	—	+	25	5.3
Others											74	15.7
Total number of strains											470	100.0

Distribution of *Klebsiella* strains according to species and antibiotic resistance pattern is shown in Table III. Strains showing resistance against ampicillin and tetracycline were most commonly observed among those showing double antibiotic resistance. This resistance pattern occurred most frequently among *K. aerogenes* strains. The majority of strains displaying resistance against three antibiotics showed a resistance pattern of ampicillin, streptomycin and tetracycline, and belonged to *K. aerogenes*. Among strains showing

Table II
Antibiotic resistance patterns of Enterobacter strains

Designation of resistance pattern	Ap	Sm	Cm	Tc	Nm	Km	Gm	Pm	Co	Nal	Total	
											No.	%
1	—	—	—	—	—	—	—	—	—	—	5	4.9
2	+	—	—	—	—	—	—	—	—	—	78	75.7
3	+	+	+	+	—	—	—	—	—	—	4	3.9
4	+	+	+	+	+	+	—	—	—	—	3	2.9
5	+	+	+	+	—	—	—	—	—	+	3	2.9
6	+	+	+	+	+	+	—	—	—	+	3	2.9
Others											7	6.8
Total number of strains											103	100.0

resistance against more than three antibiotics, coresistance of Ap Sm Cm Tc was the most frequent; it was followed with respect to frequency by a pattern of Ap Sm Cm Tc Nm Km. Other common resistance patterns were Ap Sm Cm Tc Nm Km Nal and Ap Sm Cm Tc Nal. These frequent resistance patterns were also observed among strains belonging to *K. atlantae*, *K. edwardsii*,

Table III
Antibiotic resistance pattern of strains belonging to different Klebsiella species

Designation of resistance pattern								Strains	
	<i>K. aerogenes</i>	<i>K. atlantae</i>	<i>K. edwardsii</i>	<i>K. oxytoca</i>	<i>K. ozaenae</i>	<i>K. pneumoniae</i>	<i>K. rhinoscleromatis</i>	No.	%
1	22	—	—	—	2	3	1	28	6.0
2	172	11	1	3	24	2	1	214	45.5
3	26	1	—	—	2	—	—	29	6.2
4	17	1	—	—	1	—	1	20	4.3
5	27	1	1	—	1	2	1	33	7.0
6	10	—	2	—	—	2	2	16	3.4
7	25	3	—	—	1	—	2	31	6.6
8	17	1	2	—	4	1	—	25	5.3
Others	56	7	3	—	3	3	2	74	15.7
Total number of strains	372	25	9	3	38	13	10	470	100.0

K. ozaenae, *K. pneumoniae* and *K. rhinoscleromatis*. Considering the resistance pattern, a less variegated picture was seen in *Enterobacter* (Table IV) compared to *Klebsiella*. Strains showing more than three antibiotic resistance markers occurred frequently in *E. cloacae* and *E. liquefaciens*. Strains showing resistance against 6 or 7 antibiotics (Ap Sm Cm Tc Nm Km; Ap Sm Cm Tc Nm Km Nal) belonged to *E. cloacae* and *E. liquefaciens*.

Table IV

Antibiotic resistance pattern of strains belonging to different Enterobacter species

Designation of resistance pattern	<i>E. aerogenes</i>	<i>E. cloacae</i>	<i>E. liquefaciens</i>	Total	
				No.	%
1	—	2	3	5	4.9
2	4	71	3	78	75.7
3	—	1	3	4	3.9
4	—	2	1	3	2.9
5	—	1	2	3	2.9
6	—	—	3	3	2.9
Others	—	2	5	7	6.8
Total number of strains	4	79	20	103	100.0

Frequency of R-plasmid in Klebsiella and Enterobacter strains. Incidence of R⁺ *Klebsiella* and *Enterobacter* strains are shown in Table V according to the type of resistance observed. R-plasmid was demonstrable in 2.9% of single resistant, in 12.8% of double resistant, and in 71.4% of multiple resistant *Klebsiella* strains.

Table V

Occurrence of R⁺ strains among antibiotic resistant Klebsiella and Enterobacter strains

Type of resistance	No. of R-plasmid examinations		R ⁺ <i>Klebsiella</i> strains		R ⁺ <i>Enterobacter</i> strains	
	<i>Klebsiella</i>	<i>Enterobacter</i>	No.	%	No.	%
Monoresistance	139	1	4	2.9	—	—
Double resistance	39	—	5	12.8	—	—
Multiple resistance	91	5	65	71.4	3	60.0
Total number of R-plasmids	269	6	74	27.5	3	50.0

Three strains carried R-plasmids out of 5 multiple resistant *Enterobacter* strains.

With respect to *Klebsiella* species, R-plasmids were found in 66.7% of *K. atlantae*, in 26% of *K. aerogenes* and in 20.8% of *K. ozaenae* strains (Table VI). In spite of the low number of strains examined, the observed difference can be appreciated; the difference in R-plasmid carriage between *K. atlantae* and *K. ozaenae* strains was significant ($P < 0.001$). Fewer strains were examined for R-plasmid carriage in *Enterobacter* than in *Klebsiella* species. R-plasmids could be demonstrated in *E. cloacae* and *E. liquefaciens* strains, too.

Table VI

Incidence of R⁺ strains among antibiotic resistant Klebsiella and Enterobacter species

Species	No. of strains examined	R ⁺ strains	
		No.	%
<i>K. aerogenes</i>	223	58	26.0
<i>K. atlantae</i>	12	8	66.7
<i>K. edwardsii</i>	—	—	—
<i>K. oxytoca</i>	3	—	—
<i>K. ozaenae</i>	24	5	20.8
<i>K. pneumoniae</i>	3	3	100.0
<i>K. rhinoscleromatis</i>	4	—	—
<i>E. aerogenes</i>	—	—	—
<i>E. cloacae</i>	4	1	25.0
<i>E. liquefaciens</i>	2	2	100.0

Resistance determinants of R-plasmids. Table VII shows the resistance determinants, transferred resistance determinants, and origin of the *Klebsiella* and *Enterobacter* donor strains. The majority of resistance determinants of multiple resistant *Klebsiella* and *Enterobacter* strains were transferable. Out of 25 strains resistant against 6 antibiotics in as much as 21 strains, 3 or more resistance determinants proved to be transferable.

Klebsiella strains originated from 5 different hospitals, and *Enterobacter* strains from 2 departments.

In Table VIII the resistance determinants of 442 *Klebsiella* strains are shown on the basis of whether they could be transferred totally or partially into *E. coli* K12 recipient. There were only 6 *Enterobacter* strains examined for R-plasmids. In the case of the 5 multiple resistant strains examined, only partial transfer of resistance determinants occurred in 3 cases.

Table VII

Transferred resistance determinants of Klebsiella and Enterobacter strains according to resistance determinants and origin of donors

Resistance determinants donors	No. of <i>Klebsiella</i> and <i>Enterobacter</i> strains	Transferred resistance determinants	No. of strains	Origin of strains
Ap	4	Ap	4	H.1, H.2
Ap Tc	5	Ap Tc	2	H.1, H.5
		Tc	3	H.1
Ap Sm Cm	1	Ap Sm Cm	1	H.1
Ap Sm Tc	7	Ap Sm Tc	3	H.1, H.3, H.5
		Ap Tc	2	H.1
		Sm Tc	1	H.3
		Ap	1	H.5
Ap Cm Tc	7	Ap Cm	2	H.1
		Cm Tc	2	H.1
		Ap Cm Tc	3	H.5, H.4
Ap Sm Cm Tc	14+2	Ap	1	H.5
		Sm	1	H.1
		Cm Tc	1	H.5
		Ap Cm Tc	2+1	H.1, H.3
		Sm Cm Tc	3+1	H.1
		Ap Sm Cm Tc	6	H.1, H.5
Ap Sm Tc Km Nm	1	Ap Sm Tc Km Nm	1	H.5
Ap Sm Tc Pm	1	Ap Sm	1	H.1
Ap Cm Tc Km Nm	6	Cm Tc	3	H.1
		Cm Tc Km Nm	3	H.1
Ap Sm Cm Km Nm	1	Ap Sm Cm Km Nm	1	H.2
Ap Sm Cm Tc Km Nm	24+1	Sm	1	H.1
		Cm Tc	3	H.1, H.4
		Sm Cm Tc	2	H.1
		Sm Cm Tc Km Nm	10+1	H.1, H.2, H.3, H.4, H.5
		Ap Sm Cm Tc Km Nm	8	H.1, H.2, H.3, H.4, H.5
Ap Sm Cm Tc Km Nm Gm	3	Ap Sm Cm Tc Km Nm Gm	3	H.1, H.3, H.4

H.1 = Urological ward, Budapest

H.2 = Surgical Department I, Budapest

H.3 = County Hospital, infant's ward and premature infant's ward (1)

H.4 = County Hospital, premature infant's ward (2)

H.5 = Others: different hospital wards

Table VIII

Antibiotic resistance patterns and occurrence of transferable antibiotic resistance determinants in Klebsiella strains

Antibiotic resistance determinants		No. of anti-biotic resist-ant strains	No. of strains examined	Transfer of resistance determinants		No. of trans-ferred R-plasmids
				Partial	Total	
Ap		214	139	—	4	4
Ap Sm		4	3	—	—	—
Ap Cm		9	9	—	—	—
Ap Tc		29	27	3	2	5
Ap Co		9	—	—	—	—
Ap Nal		5	—	—	—	—
Ap Sm Cm		1	1	—	1	1
Ap Sm Tc		20	12	4	3	7
Ap Sm Nal		2	—	—	—	—
Ap Cm Tc		9	9	4	3	7
Ap Cm Nal		2	—	—	—	—
Ap Km Nm		1	1	—	—	—
Ap Tc Pm		2	—	—	—	—
Ap Tc Nal		1	—	—	—	—
Ap Sm Cm Tc		33	25	8	6	14
Ap Sm Cm Nm		1	—	—	—	—
Ap Sm Cm Nal		1	—	—	—	—
Ap Sm Tc Pm		1	1	1	—	1
Ap Cm Pm Co		1	—	—	—	—
Ap Sm Tc Co		3	—	—	—	—
Ap Cm Tc Nm		1	—	—	—	—
Ap Cm Tc Km		1	—	—	—	—
Ap Cm Tc Nal		2	—	—	—	—
Ap Tc Co Nal		1	—	—	—	—
Ap Sm Cm Tc Co		1	—	—	—	—
Ap Sm Cm Nm Km		1	1	—	1	1
Ap Sm Cm Tc Nal		16	—	—	—	—
Ap Sm Tc Nm Km		3	1	—	1	1
Ap Cm Tc Nm Km		6	6	6	—	6
Ap Sm Cm Tc Nm Km		31	31	16	8	24
Ap Cm Tc Nm Km Nal		1	—	—	—	—
Ap Sm Cm Tc Nm Km Gm		3	3	—	3	3
Ap Sm Cm Tc Nm Km Nal		25	—	—	—	—
Ap Sm Cm Tc Nm Km Gm Nal		2	—	—	—	—
Total	No.	442	269	42	32	74
	%	100.0	60.9	15.6	11.9	27.5

Figure 1 represents the distribution of transferred resistant determinants compared to the total number of strains included in these experiments. Transferred determinants were Ap in 15.6%, Sm in 57.1%, Cm in 62.8%, Tc in 53.9%, Km-Nm in 65.1%, Gm in 100% and Pm in 0%.

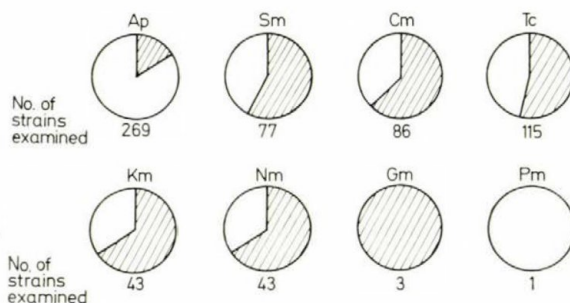


Fig. 1. Incidence of transferable antibiotic resistance determinants in *Klebsiella* strains in percentages. Shaded area: percentage of transferred antibiotic resistance determinants

Table IX

Trimethoprim resistance of Klebsiella and Enterobacter strains isolated from urine

Designation of strain	Species	Resistance determinants	MIC values of Tp $\mu\text{g/ml}$	Transferred antibiotic resistance determinants
K4	<i>K. aerogenes</i>	Ap Sm Cm Tc Km Nm Tp	1000	Sm Tc Km Nm Tp
K89	<i>K. atlantae</i>	Ap Sm Cm Tc Km Nm Tp	>1000	Sm Tp
K122	<i>K. atlantae</i>	Ap Sm Cm Tc Km Nm Tp	>1000	Sm Tp
K142	<i>K. aerogenes</i>	Ap Sm Cm Tc Km Nm Tp	>1000	Sm Cm Tc Tp
K214	<i>K. ozaenae</i>	Ap Sm Cm Tc Km Nm Tp	>1000	Sm Cm Tp
K219	<i>K. aerogenes</i>	Ap Sm Cm Tc Km Nm Tp	>1000	Cm Tc Km Nm Tp
K240	<i>K. atlantae</i>	Ap Sm Cm Tc Km Nm Tp	>1000	Sm Tp
K246	<i>K. atlantae</i>	Ap Sm Cm Tc Km Nm Tp	>1000	Sm Tp
K274	<i>K. ozaenae</i>	Ap Sm Cm Tc Km Nm Tp	>1000	Sm Tp
K397	<i>K. aerogenes</i>	Ap Sm Cm Tc Km Nm Tp	>1000	Sm Tp
K91	<i>K. atlantae</i>	Ap Sm Cm Tc Km Nm Tp	>1000	Sm Tp
K273	<i>K. aerogenes</i>	Ap Sm Cm Tc Tp	>1000	Tp
K386	<i>K. pneumoniae</i>	Ap Sm Cm Tc Tp	>1000	Ap Sm Tp
K409	<i>K. pneumoniae</i>	Ap Sm Cm Tc Tp	>1000	Ap Sm Cm Tc Tp
E.355	<i>E. liquefaciens</i>	Ap Sm Cm Tc Tp	>1000	Sm Tp
K.29	<i>K. aerogenes</i>	Ap Sm Cm Tc Tp	>1000	Sm Tp
E.24	<i>E. liquefaciens</i>	Ap Sm Cm Tc Tp	>1000	Tp
K.20	<i>K. aerogenes</i>	Ap Sm Cm Tc Tp	>1000	Sm Tp

Determination of MIC values and their relations to R-plasmids. MIC values of single and multiple antibiotic resistant *Klebsiella* strains were studied for R-plasmid carrying strains and for those in which the presence of R-plasmid could not be demonstrated. Considerably lower ampicillin MIC values were found in strains showing single ampicillin resistance (30–400 $\mu\text{g/ml}$) compared to ampicillin resistant strains showing multiple antibiotic resistance (300–2200 $\mu\text{g/ml}$). Both the low and the high types of resistance proved occasionally to be transferable.

A total of 144 multiple resistant and 58 single resistant strains belonging to the genera *Klebsiella* and *Enterobacter* was studied for trimethoprim sensitivity. Among the strains showing single antibiotic resistance (to ampicillin) there was no trimethoprim resistant strain. Among multiple antibiotic resistant cultures, as much as 33 strains (22.9%) showed resistance to trimethoprim. According to the MIC value determinations, 28 out of 33 strains proved to be resistant against 1000 $\mu\text{g/ml}$ or more trimethoprim and 5 against 40–100 $\mu\text{g/ml}$. Resistance determinant of Tp proved to be transferable into *E. coli* K12 HfrH recipient from 18 out of 33 Tp resistant strains (Table IX). Out of 15 trimethoprim resistant strains showing no transferable Tp resistance determinant, 10 showed a MIC value of 1000 $\mu\text{g/ml}$ or more and 5 ones of 40–100 $\mu\text{g/per ml}$.

Transfer frequency of R-plasmids. Using strains *E. coli* K12 HfrH and *K. pneumoniae* 43 as recipients, the transfer frequency of some R-plasmids was determined (Table X). For these experiments one representative strain of each restriction type was chosen from every hospital. In the case of multiple antibiotic resistant strains, the observed transfer frequency value was generally higher with *E. coli* K12 HfrH recipient than with the *K. pneumoniae* 43 recipient. There were two resistance determinants transferable into *E. coli* K12 strain only. Ampicillin resistance determinants of strains showing ampicillin resistance only were transferred into both recipient strains with the same frequency.

Occurrence and transfer frequency of Lac-plasmids. In the course of R-plasmid transfer experiments into *E. coli* K12 HfrH *lac*⁻ recipient strain, lactose fermenting antibiotic resistant colonies were found several times. The presence of a large number of lactose fermenting colonies allowed to conclude to Lac-plasmid uptake. In 18 out of 77 R-plasmid carrying strains was the presence of Lac-plasmid, too, observed.

In Table XI the transfer frequency of Lac-plasmids is compared with the transfer frequency of R-plasmids. From strain K15 Ap resistance determinant and Lac-plasmid as well as Tc resistance determinant and Lac-plasmid transferred together at the same frequency. Transfer of Cm resistance determinant was not accompanied with the transfer of Lac-plasmid. From strain K359 resistance determinants Ap Cm Tc and Lac-plasmid were transferred

together; the transfer frequency of Cm and Tc resistances was higher than that of Lac-plasmid; transfer of Sm and Km resistances was not accompanied with transfer of the plasmid determining lactose fermentation. All data mentioned above refer to $R^+ \text{ Lac}^+$ exconjugants. Attempts were made to obtain $\text{Lac}^+ R^-$ exconjugants; for this purpose selective media containing antibiotics were not suitable. Using the original cross experiments there were no $\text{Lac}^+ R^-$ colonies observed on nalidixic acid containing medium. Therefore, the experiments were repeated using the cellophane membrane method [18]. A total of 28 *Klebsiella* strains were tested in these cross experiments; among them, 3 donor strains generated $\text{Lac}^+ R^-$ colonies. Transfer frequencies of Lac-plasmids were 0.4×10^{-2} with strain K287 $\text{Lac}^+ R^+$, 0.7×10^{-2} with strain K279 $\text{Lac}^+ R^+$, and 2×10^{-3} with strain K288 $\text{Lac}^+ R^+$.

Table X

Transfer frequency of R-plasmids of different origin in

Designation of donor strain	Origin of strains	Material	Phage-type	Resistance determinants
K359*	H.4	nose	nt	Ap Sm Cm Tc Km Nm
K360*	H.4	throat	nt	Ap Sm Cm Tc Km Nm
K361*	H.4	throat	nt	Ap Sm Cm Tc Km Nm
K27**	H.2	urine	IV.A1	Ap
K31**	H.2	urine	IV.A1	Ap
K45**	H.2	urine	IV.A1	Ap
K15	H. others	faeces	nt	Ap Sm Cm Tc
K87	H.2	urine	V.A1	Ap Sm Cm Tc Km Nm
K88	H.2	urine	V.A1	Ap Sm Cm Tc Km Nm
K89	H.1	urine	nt	Ap Sm Cm Tc Km Nm

* same patient

** same patient

*** *E. coli* = *E. coli* K12 HfrH

K.43 = *K. pneumoniae* 43

H.1 = Urological ward, Budapest

H.2 = Surgical Department I, Budapest

H.4 = County Hospital, premature infant's ward (2)

H. others = different hospital wards

Production of colicin. Occurrence of Col-plasmid, and transfer frequency. Colicin (pneumocin) production of 314 *Klebsiella* strains was examined using 3 indicator strains. A total of 4 colicin producing strains was found. Transferability was studied using *E. coli* K12 HfrH recipient. Colicin production could be transferred to the *E. coli* strain from one (K413) out of four colicin producing strains. The observed transfer frequency was 3×10^{-2} .

Classification of R-plasmids on the basis of their fi-character. Examining the fertility inhibition character of R-plasmids of antibiotic resistant *Klebsiella* and *Enterobacter* strains, it was found that as much as 67% of 112 R-plasmids showed *fi*⁻ character, and 33% *fi*⁺ character (Table XII). Complete agreement was found between the results obtained by phage sensitivity and conjugation inhibition methods used to establish *fi*-character.

E. coli K12 HfrH and *K. pneumoniae* 43 recipient strains

Recipient strains***	Transfer frequency of resistance				
	Ap	Sm	Cm	Tc	Km
<i>E. coli</i> K.43	6×10^{-4}	1.4×10^{-1}	1.5×10^{-1} 7.5×10^{-4}	3.8×10^{-1} 1.7×10^{-3}	7.5×10^{-2} 8.4×10^{-4}
<i>E. coli</i> K.43		2×10^{-1}	6×10^{-2} 5×10^{-4}	6×10^{-2} 5×10^{-5}	5×10^{-3} 5×10^{-5}
<i>E. coli</i> K.43		2×10^{-1}	2.6×10^{-1} 1.4×10^{-4}	5.7×10^{-1} 0.7×10^{-3}	3.6×10^{-2} 0.7×10^{-4}
<i>E. coli</i> K.43	1.4×10^{-1} 1.3×10^{-1}				
<i>E. coli</i> K.43	1×10^{-2} 5×10^{-2}				
<i>E. coli</i> K.43	4.5×10^{-2} 4.5×10^{-2}				
<i>E. coli</i> K.43	1×10^{-5}		3×10^{-4} 1×10^{-4}	1×10^{-6} 1×10^{-6}	
<i>E. coli</i> K.43	2.8×10^{-3}	0.5×10^{-4} 0.5×10^{-5}	2.8×10^{-3} 5×10^{-2}	4.4×10^{-3} 3×10^{-2}	3.9×10^{-3} 5×10^{-2}
<i>E. coli</i> K.43	3×10^{-1} 1.6×10^{-2}	7.8×10^{-1} 1×10^{-4}	1.7×10^{-1} 1×10^{-2}	7×10^{-1} 1.6×10^{-3}	3.7×10^{-1} 8.8×10^{-4}
<i>E. coli</i> K.43	1×10^{-6}		5×10^{-5}	5×10^{-5}	5×10^{-5}

Table XI
Transfer frequency of R- and Lac-plasmids in *Klebsiella* → *E. coli* system

Designation of strain	Antibiotic resistance determinants	Transferred plasmids	Transfer frequency				
			Ap	Sm	Cm	Tc	Km
K15	Ap Sm Cm	R ⁺	1×10^{-5}	—	3×10^{-4}	1×10^{-6}	—
	Tc	Lac ⁺	1×10^{-5}	—	—	1×10^{-6}	—
K359	Ap Sm Cm	R ⁺	6×10^{-4}	1.4×10^{-1}	1.5×10^{-1}	3.8×10^{-1}	7.5×10^{-2}
	Tc Km Nm	Lac ⁺	6×10^{-4}	—	2.5×10^{-2}	0.8×10^{-2}	—

Table XII
Distribution of *Klebsiella* and *Enterobacter* R-plasmids according to their *fi*-character

Resistance determinants		<i>fi</i> ⁺	<i>fi</i> ⁻	Total number
Ap		1	6	7
Sm		1	4	5
Cm		—	1	1
Tc		4	—	4
Ap Sm		—	1	1
Ap Cm		1	3	4
Ap Tc		—	3	3
Sm Tc		—	1	1
Cm Tc		3	14	17
Ap Sm Cm		—	1	1
Ap Sm Tc		2	2	4
Ap Cm Tc*		4	5	9
Sm Cm Tc*		1	10	11
Ap Sm Cm Tc		3	7	10
Sm Tc Km Nm		—	1	1
Cm Tc Km Nm		1	1	2
Ap Sm Tc Km Nm		—	3	3
Sm Cm Tc Km Nm*		8	7	15
Ap Sm Cm Tc Km Nm		6	4	10
Ap Sm Cm Tc Km Nm Gm		2	1	3
R-plasmids		37	75	112
		%		
		33.0	67.0	100.0

* Plasmids of *Enterobacter* strain: Ap Cm Tc: 1 *fi*⁻
Sm Cm Tc: 1 *fi*⁺
Sm Cm Tc Km Nm: 1 *fi*⁺

Establishing incompatibility groups. Incompatibility groups of 5 R-plasmids originating from *Klebsiella* strains were determined (Table XIII). Two of them belonged to FI, one to FII and the other two of Iz incompatibility groups.

Table XIII
Grouping of Klebsiella R-plasmids according to incompatibility

Species	Designation of R-plasmid	Incompatibility group	Resistance determinants	Phage restriction
<i>K. aerogenes</i>	K16	FI	Tc	T2 T6 T7
	K310	FI	Cm Tc Km	T2 T3 T4 T5 T6 T7
	K413	FII	Tc Sm Km Gm	T1 T7
	K267	Iz	Cm Km	T2 T3 T4 T5
<i>K. ozaenae</i>	K220	Iz	Cm Tc	T1 T2 T3 T4 T5 T6 T7

Phage-restriction and phage-modification caused by R-, Lac- and Col-plasmids. Restriction of phage λ and coliphages T was studied by all the R-, Lac- and Col-plasmids demonstrated. R-plasmids from *Klebsiella* and *Enterobacter* strains were transferred to *E. coli* K12 HfrH and changes in the phage sensitivity of the recipient were studied. On the basis of their restriction on λ and T phages, altogether 14 different kinds of restricting R-plasmids and a group of non-restricting R-plasmids could be established and differentiated (Table XIV). Most R-plasmids restricted phages λ , T1, T3, T4, T5 and T6. Fifty seven R-plasmids out of 112 showed phage restriction. While among restrictive R-plasmids those with fi^+ character occurred in higher percentage than plasmids with fi^- character, among non-restrictive ones R-plasmids with fi^- character occurred in 90%. Each of the 2 R-plasmids belonging to incompatibility group FI, and each of the other two belonging to incompatibility group Iz were different in phage restriction character.

In Table XV R-plasmids are grouped according to the origin of donor strains, and data on phage restriction, resistance determinants, and phage-type of the donor strains are also given. R-plasmids carried by strains from the same hospital showed a heterogeneous restriction pattern. Strains showing the same phage-type also carried R-plasmids with different restriction types.

The stability of phage restriction patterns was checked by phage typing of a number of exconjugant colonies growing on selective plates in cross experiments.

Out of 87 R^+ exconjugant colonies deriving from the K15 R^+ strain, 37 colonies (Ap Sm Cm Tc) showed the same phage restriction. The remaining

Table XIV

Distribution of *Klebsiella* and *Enterobacter* R-plasmids according to phage restriction

No. of R-plasmids			Phage-restriction								<i>fi</i> ⁺	<i>fi</i> ⁻
			λ	T1	T2	T3	T4	T5	T6	T7		
	3		+	+	+	+	+	+	+	—	2	1
	16		+	+	—	+	+	+	+	—	14	2
	8		+	+	—	+	+	+	—	—	8	—
	1		+	+	+	—	—	—	+	+	1	—
	6*		—	+	—	+	+	+	+	—	3	3
	1		—	—	+	+	+	+	+	—	1	—
	4		—	+	—	+	+	+	—	—	—	4
	4		—	—	—	+	+	+	+	—	—	4
	1		—	+	—	+	—	—	+	—	—	1
	3		—	—	—	+	+	+	—	—	—	3
	1		—	+	—	+	+	—	—	—	—	1
	7		—	—	—	+	+	—	—	—	2	5
	1		—	+	—	—	—	—	+	—	—	1
	1		—	—	—	—	+	—	—	—	—	1
Restrictive	No.	57									31	26
	%	50.9									54.4	45.6
Not restrictive	No.	55**									6	49
	%	49.1									10.9	89.1
Total	No.	112									37	75
	%	100.0										

* Plasmid of an *Enterobacter* strain (*fi*⁻)** Plasmids of two *Enterobacter* strains (one of them *fi*⁻, the other *fi*⁺)

50 exconjugant colonies carrying only the R-plasmid coding for Cm Tc resistances showed another type of phage restriction. In the case of the K89 donor strain there were four types of resistance patterns (Sm Cm Tc Km; Cm Tc Km; Tc Km; Tc) among the 117 colonies tested, but all of them showed the same phage sensitivity.

After transferring R-plasmid from a multiple antibiotic resistant (Ap Sm Cm Tc Tp resistant) strain (K142) into *E. coli* K12 HfrH recipient, phage restriction was studied in 60 different exconjugant colonies. In 32 colonies (showing Ap Sm Cm Tc; Ap Sm Nm-Km resistance patterns) presence of non-restrictive *fi*⁻ R-plasmid, and in 28 colonies (showing Sm Cm Tc; Sm Cm Tc Tp; Sm Tc Tp; Cm Tc Tp and Cm Tp resistance patterns) presence of *fi*⁺ R-plasmid causing the same phage restriction pattern was demonstrated.

Fifty one R-plasmids were examined for their hsp II phage restriction. In Table XVI data of three plasmids showing hsp II restriction are shown. As many as 48 R-plasmids were found not to determine hsp II phage restriction.

Table XV

Distribution of *Klebsiella* and *Enterobacter* R-plasmids according to restriction of λ and T phages, *fi*-character and the origin of R⁺ strains

Origin of R ⁺ strains	Phage-restriction								No. of resistance determinants	<i>fi</i>		No. of R ⁺ strains	Phage type of R ⁺ strains
	λ	T1	T2	T3	T4	T5	T6	T7		+	—		
H.1 Urinary tract infections	+	+	—	+	+	+	+	—	1-5	4	2	6	IIA1, VIIA1, nt
	+	+	—	+	+	+	+	—	1-5	3	—	3	nt
	—	+	—	+	+	+	+	—	2-5	2	2	4	nt
	—	+	—	+	+	+	—	—	2-4	—	4	4	nt
	—	—	—	+	+	+	+	—	2-3	—	4	4	nt
	—	+	—	+	+	—	—	—	2	—	1	1	nt
	—	+	—	+	—	—	+	—	2	—	1	1	nt
	—	—	—	+	+	+	—	—	2-3	—	3	3	nt
	—	—	—	+	+	—	—	—	1-5	—	3	3	VIIA1, nt
	—	—	—	—	—	—	—	—	1-5	2	20	22	IIA1, IVA1, nt; C14*
H.2 Urinary tract infections	+	+	+	+	+	+	+	—	3	—	1	1	nt
	—	+	—	+	+	+	+	—	3-5	1	1	2	nt
	—	—	—	—	—	—	—	—	1-6	—	8	8	IIIA1, IVA1, VA1, K35, nt; C14*
H.3 Alimentary and respiratory tract infections	+	+	+	+	+	+	+	—	1-5	2	—	2	nt
	—	—	+	+	+	+	+	—	1	1	—	1	nt
	—	—	—	+	+	—	—	—	2-6	2	2	4	nt
	—	—	—	—	—	—	—	—	3-4	2	6	8	nt
H.4 Respiratory tract infections	+	+	—	+	+	+	+	—	5-6	10	—	10	nt
	+	+	—	+	+	+	—	—	5	2	—	2	IA1, nt
	—	+	—	—	—	—	+	—	2	—	1	1	nt
	—	—	—	—	—	—	—	—	3-6	—	2	2	nt
H.5 Alimentary, respiratory and urinary tract infections	+	+	—	+	+	+	—	—	2-4	3	—	3	K1 4, 7, nt
	+	+	+	—	—	—	+	+	3	1	—	1	nt
	—	—	—	—	+	—	—	—	3	—	1	1	nt
	—	—	—	—	—	—	—	—	1-6	2	13	15	IVA1, VIIIA2, K4
Total										37	75	112	

* *Enterobacter*

H.1 = Urological ward, Budapest

H.2 = Surgical ward, Budapest

H.3 = County Hospital, infant's and premature infant's ward (1)

H.4 = County Hospital, premature infant's ward (2)

H.5 = Different hospital wards

Table XVI
Restriction of phage λ vir by different Klebsiella R-plasmids

Plasmids	Resistance	E.o.p. of phage λ vir propagated on <i>E. coli</i> strain	
		J53	J53(N3) ⁺
N3	Sm Tc	4×10^{-4}	1
R350	Ap Cm	1.8×10^{-1}	1
R13	Ap Cm Tc	1.2×10^{-3}	1
R142	Sm Cm Tc	2×10^{-4}	1

Phage restriction and phage modification capacity of *Klebsiella* R-plasmids was studied on phages Plkc, λ vir, T1, T3 and T4. The data for 3 plasmids are shown in Table XVII.

Both the R⁺ Lac⁻ and the R⁺ Lac⁺ *E. coli* K12 HfrH exconjugant colonies of six R⁺ Lac⁺ donor strains showed the same phage restriction pattern indicating that phage restriction may be attributed to the uptake of R-plasmids instead of Lac-plasmids.

After crossing strain K413 R⁺ Col⁺ (Ap Sm Tc Nm Km) with *E. coli* K12 HfrH lac⁻ recipient strain, out of 50 colonies picked from the colicin containing plates 17 proved to be Col⁺ R⁺ and 33 Col⁺ R⁻. The observed restriction patterns were the same with both types of colonies, 17 Col⁺ R⁺ and the 33 Col⁺ R⁻. According to these data phage restriction may sometimes be attributed to the presence of Col-plasmid instead to the presence of R-plasmids (the K413 R⁺ Col⁺ donor strain was not typable).

Relation of the presence of R-plasmids to the phage-type of Klebsiella strains.
 As many as 27.5% of 269 *Klebsiella* strains examined for R-plasmid carriage proved to carry R-plasmid.

In Table XVIII, strains with different phage-types are shown according to their R-plasmids. In view of the few strains belonging to individual phage-types, it was not possible to conclude to a relation between phage-types and R-plasmid carrier state. Nevertheless, a considerable difference was found in the R-plasmid carrier state of typable and not typable strains. Approximately one half of the typable strains and 4/5 of the non-typable ones proved to be R-plasmid carrier, the difference was significant ($P < 0.05$).

R-plasmids from 57 *Klebsiella* strains (causing phage restriction in *E. coli* recipient) were transferred into the antibiotic sensitive, phage-type XA1 *K. pneumoniae* 43 recipient strain. A decrease in the e.o.p. value of *Klebsiella* phage X was observed with 22 of the 57 R-plasmids. In these cases the recipient strain did not lyse with the RTD dilution of phage X and so has become non-typable.

Table XVII

Phage restriction and phage modification by Klebsiella R-plasmid R13

Strain	Restriction					Modification				
	Relative e.o.p. value of phage									
	P1ke	λ vir	T1	T3	T4	P1ke.R13	λ vir.R13	T1.R13	T3.R13	T4.R13
J53 R ⁻	1	1	1	1	1	1	1	1	1	1
J55 R13	1×10^{-4}	1×10^{-3}	1×10^{-4}	0.2×10^{-4}	0.1×10^{-4}	1	1	1	1	1
J53 R142	0.6×10^{-2}	0.1×10^{-3}	1×10^{-5}	0.6×10^{-3}	1×10^{-4}	1	1	1	1	1
J53 R187	0.3×10^{-5}	1	1×10^{-8}	0.6×10	2×10^{-1}	*	1	*	1	1

* Not examined

Table XVIII

Occurrence of multiple resistance and R-plasmids among Klebsiella strains of different phage-types

Strains	Phage-type															Typable	Non-typable	Total number of strains
	I A1	II A1	III A1	IV A1	V A1	VI A1	VII A1	VIII A1	IX A1	X A1	XI A1	XII A1	XIII A1	XIV A1	XV A1			
No. of phage typed strains	11	91	5	21	4	2	13	17	—	1	—	1	—	—	3	14	183	470
No. of multiresistant strains	8	8	4	6	3	2	6	4	—	—	—	—	—	—	—	9	122	172
No. of strains examined for R-plasmid carriage	3	7	3	5	3	1	5	4	—	—	—	—	—	—	—	9	51	91
No. of R ⁺ strains	1	7	1	3	3	—	2	2	—	—	—	—	—	—	—	3	43	65

Table XIX
Transfer frequency of Klebsiella R-plasmids in vitro and in vivo using

Donor strain	Resistance determinants	Recipient strain	Experimental system
K359	Ap Sm Cm Tc Km Nm	<i>E. coli</i> K12 C600 F ⁻	<i>in vitro</i> <i>in vivo</i>
K359	Ap Sm Cm Tc Km Nm	<i>K. pneumoniae</i> 43	<i>in vitro</i> <i>in vivo</i>
K31	Ap	<i>E. coli</i> K12 C600 F ⁻	<i>in vitro</i> <i>in vivo</i>
K31	Ap	<i>K. pneumoniae</i> 43	<i>in vitro</i> <i>in vivo</i>

Comparison of transfer frequency of R- and Lac-plasmids in vitro and in vivo. A single antibiotic resistant strain, K31 (Ap), was able to transfer its resistance determinant to *E. coli* K12 C600 F⁻ and *K. pneumoniae* 43 recipient strains in mouse experiments *in vivo*. In the case of *E. coli* recipient, a lower frequency of transfer was observed *in vivo* than *in vitro*. In the case of *Klebsiella* recipient, the frequency of transfer was the same *in vitro* and *in vivo*.

All the 5 antibiotic resistance determinants (Ap Sm Cm Tc Km) of the multiple resistant strain were transferred in transfer experiments *in vivo* and *in vitro* to *E. coli* K12 C600 recipient, while only four out of five resistance determinants could be transferred *in vitro* and *in vivo* into *K. pneumoniae* 43 recipient (Table XIX).

The data described above refer to the method carried out with nalidixic acid dilution. In the case of direct spreading on eosin methylene blue agar and subsequent replication, R-plasmid transfer from the multiple resistant strain was observed to *E. coli* K12 C600 F⁻ recipient only. The lowest frequency of transfer to *E. coli* C600 F⁻ recipient both *in vitro* and *in vivo* were observed for Ap resistance (5×10^{-3}). The same low frequency of transfer was recognized for Tc resistance *in vivo*. Transfer frequencies of Sm Cm Tc Km resistances were higher *in vitro* than *in vivo*, although they were high in the latter system as well. With the recipient strain *K. pneumoniae*, 43 transfer frequencies of Sm Cm Km resistance determinants were lower than those observed with *E. coli* K12 C600 F⁻ recipient. In the *in vivo* system, transfer of only Sm and Km resistance determinants took place at lower frequency than in the *in vitro* system.

Transfer frequency of R- and Lac-plasmids in vitro and in vivo. *Klebsiella aerogenes* strain K359, an R- and Lac-plasmid carrier, was crossed with *E. coli*

E. coli K12 C600 F⁻ and *K. pneumoniae* 43 strains as recipients

Ap	Transfer frequency of resistance				Phage restriction
	Sm	Cm	Tc	Km	
5×10^{-3}	5×10^{-4}	7×10^{-1}	5×10^{-1}	5×10^{-1}	+
0.5×10^{-3}	0.1×10^{-2}	1.7×10^{-2}	1.2×10^{-3}	0.1×10^{-2}	+
—	3×10^{-4}	7.5×10^{-4}	1.7×10^{-3}	3×10^{-4}	+
—	0.4×10^{-6}	6.6×10^{-4}	0.3×10^{-3}	0.4×10^{-6}	+
9×10^{-1}					—
1×10^{-2}					—
5×10^{-2}					—
0.8×10^{-2}					—

K12 C600 F⁻ and the transfer frequency of both plasmids was examined *in vitro* and *in vivo*. From the multiple resistant donor strain (Ap Sm Cm Tc Km) all but the Tc resistance determinants were transferred together with the Lac-plasmid *in vitro*. Transfer frequency of R-plasmid proved to be higher (5×10^{-3} , 5×10^{-1} , 7×10^{-1} , 5×10^{-1}) than that of Lac-plasmid (3.5×10^{-4} , 0.7×10^{-4} , 0.2×10^{-3} , 1.4×10^{-4}). In experiments *in vivo*, all R-determinants could be transferred. Lac-plasmid was transferred together with Ap and Cm resistance determinants only. In these *in vivo* experiments the observed transfer frequencies of Lac-plasmid were also lower (0.5×10^{-4} , 0.8×10^{-4}) than the R-plasmid transfer frequencies (0.5×10^{-3} , 0.1×10^{-2} , 1.2×10^{-3} , 0.2×10^{-2}).

Phage restriction capacity of R-plasmids transferred in vitro and in vivo. The multiple resistance determining R-plasmid K359 caused T3, T4 and T6 phage restriction transferring it into *E. coli* K12 C600 F⁻ recipient both *in vitro* and *in vivo*. Disregarding minute differences, the observed e.o.p. values proved to be the same.

Discussion

Strains of the genus *Klebsiella* are much more frequently isolated as causative agents in hospital epidemics than strains belonging to the genus *Enterobacter*. Presence of R-plasmids in *Klebsiella* strains is also more frequently mentioned in the literature than in *Enterobacter* strains.

TKACZOWA *et al.* [23] reported that they found most frequently Sm single antibiotic resistant strains (Ap was not included in their examinations), more rarely Pc, Cm and Tc single resistant strains, whereas Sm Cm Tc multiple resistant strains were found in only 3.6% among the examined *Klebsiella* strains. Strains resistant to Nm were found in 2.9%.

In our study, strains resistant to Ap only were the most frequent (45.5%) among strains belonging to the genus *Klebsiella*. Among resistance patterns including more than one resistance markers, the Ap Sm Cm Tc and Ap Sm Cm Tc Nm-Km patterns were the most frequent; both of them became general in strains of *K. aerogenes*.

Predominance of strains resistant against Ap only was also observed (75.5%) among those belonging to *Enterobacter*. The most frequent was the Ap Sm Cm Tc resistance pattern (in *E. liquefaciens*). As many as 34 different resistance patterns were found among *Klebsiella*, and 10 among *Enterobacter* strains.

MIC values of Ap resistant strains showing single or multiple resistance were found to be different. For the single resistant strains a low MIC value was found. Resistance determinants of the 30 single resistant (against Ap) strains examined for MIC proved to be not transferable, reflecting probably a chromosomal localization of the resistance determinants in these strains. The Ap resistance could be transferred from 12 out of 28 multiple resistant including Ap resistant strains.

Among strains originating from a urological ward, THIEMKE and NATHAN [24] found that the strains *K. pneumoniae* K2 and K30, isolated during the same hospital outbreak, differed in their MIC values. They suggested a determination of MIC values for epidemiological purposes, in addition to serotyping. This suggestion was not confirmed by our MIC value determinations as different MIC values were found for strains isolated in the same hospital, carrying the same resistance determinants probably on the same R-plasmids.

R-plasmids were demonstrated in 71.4% of multiple resistant *Klebsiella* strains, and a considerably lower transferability was observed for resistance determinants of double (12.8%), and single (2.9%) resistant strains. R-plasmid examinations were carried out with strains belonging to all species of *Klebsiella* except *K. edwardsii*. R-plasmid carriage was demonstrated in 27.5% of the 269 strains examined. Frequent R-plasmid carriage was observed with *K. pneumoniae* and *K. atlantae*.

Considerably different results have been published on R-plasmid carriage of hospital *Klebsiella* strains in different countries. SMITH and ARMOUR [25] who studied the transferable resistance of enteric bacteria of urinary tract origin in Boston, found the occurrence of R-plasmids in a particularly high percentage, as 6 out of 10 *Klebsiella* strains proved to be plasmid carriers, and 69% of all enteric bacteria showed transferable antibiotic resistance. AANDAHL in Norway [26] found R-plasmids in 45% of the strains. In the USA, HINSHAW *et al.* [27] demonstrated the presence of R-plasmids in 4.1% of *Klebsiella* strains. According to ANDERSON *et al.* [28], 39% of hospital *Klebsiella* strains were plasmid carriers. Using *S. typhi* as recipient strain,

DZIERZANOWSKA [29] was able to demonstrate the presence of R-plasmids in 90% of antibiotic resistant *Klebsiella* strains.

SMITH and PARSELL [30] examined the *in vivo* plasmid transfer of Lac- and R-plasmid carrying *Klebsiella* strains in chickens. Plasmids of Lac⁺ R⁺ *Klebsiella* strains could be transferred to *S. typhi-murium* and *S. gallinarum* strains by giving the chickens Ap containing food. The diagnostic significance of Lac-plasmid carrying strains was emphasized by the authors. Lac-plasmids were frequently found in *Klebsiella* strains in the course of our study (in 18 out of 77 R⁺ strains) even if the search for Lac-plasmid carrying colonies was carried out by selection for antibiotic resistance.

Lac-plasmids code for quantitatively and qualitatively different galactosidases, e.g. in a Cm Sm resistant R⁺ *K. aerogenes* strain a plasmid coded a β -galactosidase enzyme exceeding the average enzyme activity [31].

In the course of our studies, multiple resistance was more frequently transferable to the *E. coli* K12 recipient than in the *K. pneumoniae* 43 (10^{-1} to 10^{-6} in the case of the individual resistance determinants), in agreement with the observation of SALZMANN and KLEMM [32]. From a *Klebsiella* strain of clinical origin, which showed resistance against 10 antibiotics, R-plasmid was transferred at a frequency of $4-5 \times 10^{-3}$ by TRAUB and KLEBER [33].

The single Ap resistance could be transferred only from four of the 139 strains tested. In cases of unsuccessful transfer experiments, efforts were made to transfer resistance determinants repeating the transfer experiment several times, but no more R-plasmid could be demonstrated. It is believed that the repeatedly unsuccessful experiments point to a chromosomal localization of the resistance determinants. Ap resistance determinant of multiple antibiotic strains could be transferred in 40.2% and it is thought to be linked to a chromosome or to a non-transferable plasmid in these strains. MADEIROS and O'BRIEN [34] and NAIDE *et al.* [35] also found that Ap resistance was rarely transferable, and this was attributed mostly to its chromosomal localization in the strains.

Except for Ap resistance, all other resistance determinants (Sm Cm Km-Nm) could be transferred more frequently (in 57.1-65.1%) than the Tc resistance determinant (53.9%). Similar observations was done by HINSHAW *et al.* [27] concerning *Klebsiella* R-plasmids.

Nearly one half of the multiple resistant *Klebsiella* and *Enterobacter* strains showing Tp resistance proved to carry transferable Tp resistance; 27 out of the 33 strains tested showed a Tp MIC value of 1000 $\mu\text{g/ml}$ or more, and of these 18 strains carried transferable Tp resistance. According to DATTA and HEDGES [11] *Klebsiella* strains showing a Tp MIC value of 1000 $\mu\text{g/ml}$ possessed transferable Tp resistance and carried a plasmid belonging to incompatibility group W. In ten multiple resistant strains in our material, Tp resistance determinant conferring an MIC value of 1000 $\mu\text{g/ml}$ was not transferable

like the other resistance markers. These strains were considered to have lost their transfer factor.

In our material the 112 R-plasmids tested proved to be fi^- in 67% and fi^+ in 33%.

IYER and IYER [36] gave a detailed characterization of an R-plasmid derived from a phage type IIA1 *Klebsiella* strain isolated in Hungary. This Ap Sm Cm Tc Nm and Km resistance determining R-plasmid showed fi^+ character.

Five plasmids examined for incompatibility in our study belonged to incompatibility groups of FI, FII and Iz.

According to DATTA [37] R-plasmids of *Klebsiella* strains belong to incompatibility groups of FI, FII, I, P and W. R-plasmids belonging to incompatibility group C were described by MIZON *et al.* [38].

As many as 54.4% of the R-plasmids coding for phage restriction proved to be fi^+ , and only 10.9% of R-plasmids causing no phage restriction proved to be fi^+ . These data nearly coincide with those of LÁSZLÓ and RIMANÓCZY [39] and MILCH *et al.* [40] on fi -characters of phage restricting R-plasmids of enteric bacteria isolated in Hungary.

There are contradictory data in the literature on phage restriction of fi^+ R-plasmids. Only fi^- R-plasmids causing phage restriction were reported by WATANABE *et al.* [41]. The phage restriction capacity of both fi^+ and fi^- R-plasmids was recognized by SICCARDI [42]. In the same way both plasmids with fi^+ and fi^- character were found to cause phage restriction by BANNISTER and GLOWER [4], and YOSHIMORI *et al.* [43].

In an R-plasmid carrying strain two kinds of phage restricting exconjugant colonies were found during examinations for stability of phage restriction. Our supposition that different phage restriction patterns refer to presence of different plasmids in the strains could not be corroborated sufficiently.

Three out of 51 *Klebsiella* R-plasmids coding for phage restriction showed phage modification capacity as well. According to ROULLAND-DUSOIX *et al.* [44], R-plasmids of enteric bacteria have genes for phage restriction and phage modification in 10 to 20% of the strains.

Data on R-plasmid transfer experiments *in vivo* are rather contradictory, owing to several different factors such as different donor strains, different biological traits of different test animals, usage of pretreatment or lack of it, etc. In the course of our studies, mice were pretreated with streptomycin to exclude any effect on the transfer of the original enteric flora.

R-plasmids of the single and multiple resistant *Klebsiella* strains examined could be transferred *in vivo* to *E. coli* and *Klebsiella* recipient strains (Table XIX).

The successful experiments of SMITH [45] may be explained by the large number of donor and recipient strains used. Transfer between *Shigella* and

E. coli strains was reported by AKIBA *et al.* [46] in mice, and LY KAGIWADA *et al.* [47] in human volunteers, but without any quantitative characterization. In mouse experiments, WATANABE [15] observed no transfer after giving orally the R-plasmid carrying strain *E. coli* CSH-2. Similar results were reported by FALKOW [48], but on repeating the transfer experiments after feeding with streptomycin he could demonstrate R-plasmid carrying strains in 9 out of 10 mice. In the same way, R-plasmid transfer was achieved by giving antibiotics by KASUYA [49] and GUINÉE [50], or by using germ-free animals by SALZMANN and KLEMM [51]. Because of the presence of several inhibitory factors, WIEDEMANN [52] considers *in vivo* transfer to be a rare event; these factors, however, can be controlled by antibiotics. On the basis of our experiments, the latter circumstance is thought to be decisive. Our results concerning the demonstration and characterization of R-plasmids allow to conclude to the different origin of individual infections. This may have considerable importance in infection with *Klebsiella* strains belonging to frequent phage-types or not typable. In these cases R-plasmids have the role of an epidemiological marker.

R-plasmid conferring gentamicin resistance was demonstrated by MARTIN *et al.* [53] in *Klebsiella* strains associated with hospital infections. By characterizing the R-plasmid it was possible to pursue the spread of the infection.

Observing the serotype and the Gm resistance marker of *Klebsiella* strains, HOLZMAN *et al.* [54] examined the spread of infections and concluded that infected hands of the staff have an important role. The presence of *Klebsiella* strains on the hands of the hospital staff was demonstrated more frequently than the presence of other coliforms.

Our studies point to the fact that R-plasmid carrying *Klebsiella* strains have a dangerous role not only as causative, or potential causative, agents, but as R-plasmid reservoirs as well.

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CHARACTERIZATION OF A PARVOVIRUS STRAIN ISOLATED FROM HUMAN ADENOVIRUS TYPE 12

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A parvovirus strain was isolated from type 12 human adenovirus. The parvovirus multiplied without helper virus in HEp-2 cell cultures, while its multiplication was enhanced by type 12 and type 18 adenoviruses. Haemagglutinating infective virions as well as soluble haemagglutinins were demonstrated by ultracentrifugation and gel filtration. Virus haemagglutination inhibiting antibodies were found in the sera of healthy rats, whereas sera of healthy persons did not contain antibodies. The parvovirus failed to cause illness in laboratory animals (hamster, mouse, guinea pig).

Small virus particles of icosahedral symmetry, approximately 27 nm in diameter, were observed in a type 12 human adenovirus strain, as reported in a previous paper [1]. The virus multiplied in the nucleus of infected cells, agglutinated at low temperature intensively human and guinea pig red blood cells (RBC) and was heat resistant. In CsCl gradient the virion was found partly in the fraction of 1.4 g/cm³ density, partly together with the adenovirions in the fraction of 1.34 g/cm³ density. On the basis of these characteristics the virus strain was considered to belong to the parvovirus family.

Adenovirus-free parvovirus was successfully produced by heat treatment and propagated in HEp-2 cell culture. The strain was called PVSz. Certain biological and physico-chemical properties of this isolated virus strain are described in the present work.

Materials and methods

Cell cultures, virus strains. HEp-2 monolayer cultures were used for the propagation of the adenovirus and PVSz strains under conditions described earlier [1]. Type 12 adenovirus strain designated Ad 12P was obtained from Dr. H. G. PEREIRA (National Institute for Medical Research, Mill Hill, London). The type 12 human adenovirus strain (Ad 12K) which contained the parvovirus was obtained from Dr. K. KÖHLER (Max Planck Institut, Tübingen). Adenovirus-free parvovirus strain was prepared from the Ad 12K strain by heat treatment at 60 °C for 60 min followed by neutralization with Ad 12P immune serum [1]. Type 18 human adenovirus was obtained from Dr. R. WIGAND (Institut für Hygiene und Mikrobiologie der Universität des Saarlandes, Homburg, Saar).

Haemagglutination (HA) and haemagglutination inhibition (HI) were carried out in Takátsy's microtitrator as described in a previous publication [1]. As about 70% of untreated human serum specimens inhibited the HA with PVSz, all sera used in the HI test were treated with kaolin according to MOORE's technique [2] to eliminate aspecific HA inhibiting material.

Animal inoculation. PVSz was inoculated subcutaneously, intraperitoneally, intracerebrally and intrapulmonally into newborn and 3-month-old hamsters, subcutaneously and intraperitoneally into newborn and 2-month-old mice and guinea pigs. With intrapulmonary and intracerebral inoculation the input multiplicity was $5 \times 10^{6.5}$, in the other cases $10^{7.5}$ TCID₅₀.

Cytological examination. Histological sections were prepared from the organs of virus-inoculated animals by formalin fixation, paraffin embedding and haematoxylin-eosin staining.

Ultracentrifugation was carried out partly with the method described earlier [1] partly with the technique of MATSUNAGA et al. [3].

Gel-filtration was performed on Sephadex G-200 (Pharmacia) gel, 0.01 M phosphate buffer pH 7.0 with 0.5 M NaCl and 0.02% sodium azide content was used as eluent. Fractions were collected with an LKB Ultrarac Fraction collector and their UV absorption was registered by an Uvicord II recorder. Dextran blue, bovine serum albumin, crystallized ribonuclease and phenol red were used for calibration of the column.

Results

Multiplication of PVSz in tissue culture. HEp-2 cells were infected with PVSz separated from the human adenovirus type 12K strain. 0.1 TCID₅₀/cell was used for inoculation and the infective titre of the cultures was determined at different intervals during incubation. Values in Fig. 1 show extra- and intracellular virus yield. The virus titre showed an expressed rise up to 48 h after the infection, subsequently the rate of multiplication became moderate until the 8th day of examination.

Helper effect of adenoviruses on the multiplication of PVSz. To study the helper effect of adenoviruses, identical quantities (0.1 TCID₅₀/cell) of PVSz and different quantities of type 12 and 18 adenoviruses were inoculated simultaneously into HEp-2 cultures. The titre of PVSz was determined after 7 days incubation. As shown in Table I, both adenovirus types potentiated the replication of the parvovirus strain.

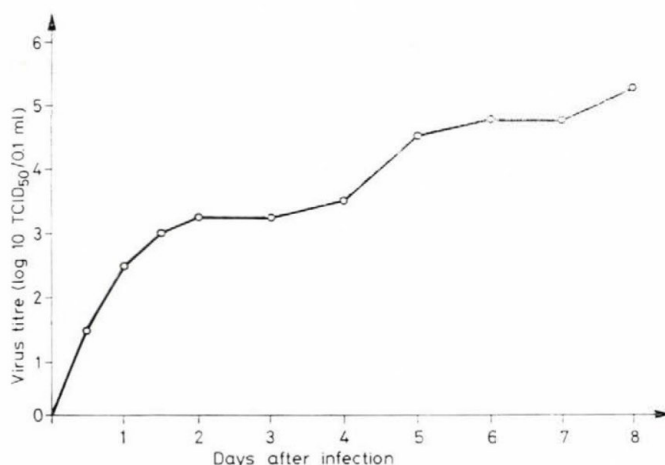


Fig. 1. Multiplication of PVSz in Hep-2 cells; input multiplicity: 0.1 TCID₅₀/cell

Table I
Effect of adenovirus on the multiplication of PVSz

Adenovirus		PVSz*
Type	Multiplicity TCID ₅₀ /2 × 10 ⁵ cell	Infective titre on the 7th day of incubation (TCID ₅₀ /0.1 ml)
12P	10 ^{2.5}	10 ^{7.5}
	10 ^{1.5}	10 ^{6.5}
	10 ^{0.5}	10 ^{5.5}
18	10 ¹	10 ^{5.75}
	10 ⁰	10 ⁵
	10 ⁻¹	10 ^{4.5}
		10 ^{4.75}

* PVSz input multiplicity 0.1 TCID₅₀/cell

Separation of PVSz haemagglutinins by gel filtration. The parvovirus was found to possess two different haemagglutinins in anion exchange chromatography [1]. Two different haemagglutinins were demonstrated also by the exclusion chromatography of PVSz on Sephadex G-200 gel column (Fig. 2). One of them appeared in the void volume while the other close to the total volume.

Ultracentrifugation of PVSz in CsCl isopycnic gradient. Using the method of MATSUNAGA *et al.* [3], haemagglutinating activity was found with CsCl isopycnic gradient centrifugation not only in the fractions of 1.40 and 1.34

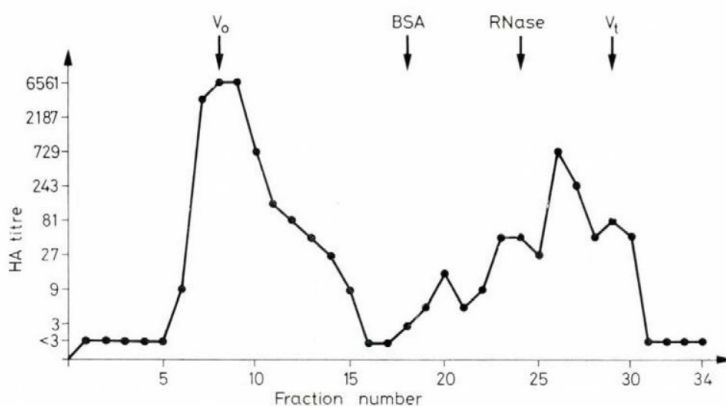


Fig. 2. Gel-filtration of PVSz on Sephadex G-200 column, using as eluent a 0.5 M NaCl containing phosphate buffer pH 7.0. Arrows indicate the void volume (V_0), the elution maxima of bovine serum albumin (BSA), and crystallized ribonuclease (RNase), and the total volume (V_t)

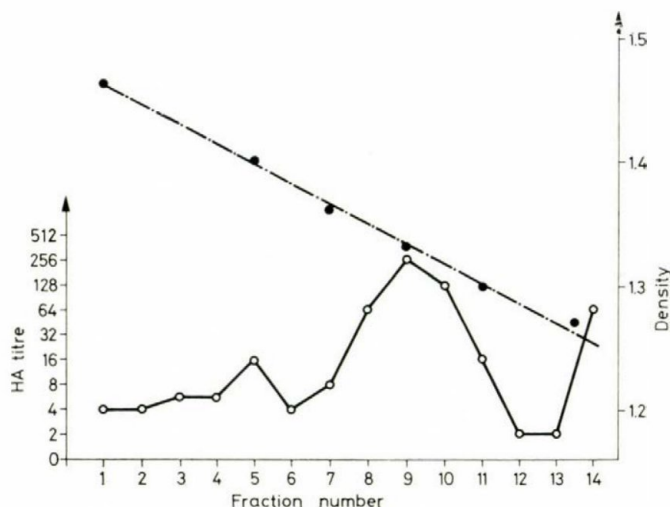


Fig. 3. Haemagglutinating activity of the fractions obtained with CsCl gradient centrifugation

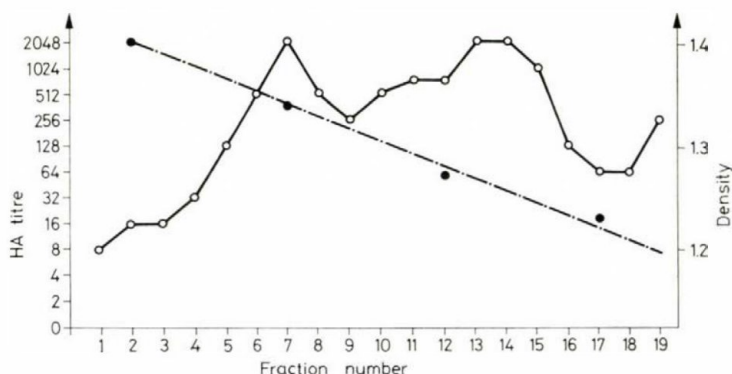


Fig. 4. Forty-eight hours CsCl density gradient centrifugation of the 1.2 g/cm³ dense haemagglutinin of PVSz. ○—○ HA titre, ●—● density (g/cm³)

g/cm³ density as demonstrated earlier [1], but also in the top fraction of the tube (Fig. 3). Subjecting the low-density fraction to repeated centrifugation in CsCl for 48 h, the density of the haemagglutinin was determined and found to be approximately 1.25 g/cm³ (Fig. 4).

Occurrence of PVSz antibodies in human and animal sera. The occurrence of antibodies against the PVSz strain was studied with HI test in sera obtained from 18 healthy persons. Eight HA units of virus and human O Rh+ RBC were used in the experiments. None of the sera inhibited the HA of the PVSz strain. Table II demonstrates the HI titres obtained with the kaolin treated sera of 13 rats and 20 pigs. Rat sera contained different amounts of antibody whereas in pig sera their quantity was negligible.

Table II
HI antibodies to PVSz in rat and pig sera

Species	No. of sera	HI titre
Rat	1	10 240
	2	5 120
	2	1 280
	2	640
	1	320
	1	160
	2	80
	2	20
Pig	4	40
	16	< 20

Inoculation of animals with PVSz. Thirty-eight mice, 35 hamsters and 6 guinea pigs were inoculated with the PVSz virus. Histological sections and homogenates were prepared from the liver, spleen, lung, kidney and brain of a group of animals, 10, 20 and 40 days after inoculation and the homogenates were placed on HEp-2 cultures. No cytopathic effect was observed in the cell cultures during 30 days incubation and the HA test of the super-

Table III
HI titre in sera of animals 7 months after inoculation with PVSz

Age and species of animals	Route of inoculation							
	Subcutaneous		Intrapulmonary		Intraperitoneal		Intracerebral	
	No. of animals	HI titre	No. of animals	HI titre	No. of animals	HI titre	No. of animals	HI titre
One-day-old mice	9	< 20			6	< 20		
	4	20			4	20		
Two-month-old mice	2	< 20			1	40	1	< 20
					1	80		
One-day-old hamster	1	160	1	160	1	320		
	2	320						
Three-month-old hamster	1	320						
Guinea-pig	1	160			1	320		

natant gave a negative result. Except for a slight lymphocytic infiltration, no other pathological alterations were revealed in the organs.

The other group of the inoculated animals observed for 7 months did not display any pathological symptoms during this period. Subsequently, blood samples were taken of the animals and they were dissected. No pathological changes were found. Table III demonstrates the results obtained in HI test with kaolin treated sera. Significant HI titres were found in the sera of guinea pigs and hamsters. Among the mice, those subjected to intraperitoneal inoculation at the age of 2 months displayed slight antibody production.

Discussion

The parvovirus strain (PVSz) was isolated from type 12 human adenovirus material (Ad 12) and propagated in HEp-2 cell cultures [1]. Though the PVSz was observed together with an adenovirus strain, it does not belong to the adeno-associated virus group, being able to replicate without the presence of another virus. Its reproduction rate (Fig. 1) does not differ from that of other parvoviruses [4, 5]. Propagated together with type 12 and type 18 human adenoviruses, the virus yield was higher (Table I). The "helper" effect of adenovirus is known also with other non-defective parvovirus strains [6].

The morphological [1], biological and physico-chemical properties of PVSz indicate that the virus belongs to the parvovirus genus of the *Parvoviridae* family [7-11]. The PVSz displayed, however, a deviating HA activity as compared to the parvoviruses known so far [1]. The PVSz fails to agglutinate rat RBC which precludes the possibility of its belonging to the "hamster osteolytic" group of the parvoviruses which includes the Kilham rat, H-1, H-3, RTV, HB, X₁₄ viruses [10, 12]. The MVM, PPV, KBSH, TVX as well as the HT and HER₅₂ parvoviruses show deviating HA characteristics [13]. On the basis of its HA activity, the isolated parvovirus resembled the Kirk and HS-3 viruses [1, 14]. Serological examinations by M. D. HOGGAN indicate likewise that the structure of the PVSz is related antigenically to the Kirk and HS-3 viruses.

In previous studies it has been shown that the density of infective PVSz virions is about 1.4 and 1.34 g/cm³ in CsCl density gradient centrifugation and they possess HA activity. The present experiments revealed in the 1.25 g per cm³ density fraction another haemagglutinin type of lower density (Figs 3 and 4) which might correspond to a soluble component. The two haemagglutinin types were demonstrated with gel-filtration (Fig. 2), too, where one of the haemagglutinins appeared together with the void volume corresponding assumably to the virion. Elution of the other haemagglutinin, occurring only

in the fractions preceding the total volume, indicates that it is a haemagglutinin of low molecular weight. The PVSz strain failed to be pathogenic for the laboratory animals studied, but HI antibodies appeared in part of the animals (Table III). Sera of healthy rats contained HI antibodies against PVSz (Table II). The presence of antibodies refers to an assumable previous infection of the rats with Kilham rat virus [15]. According to the personal communication of M. D. HOGGAN, the PVSz shows a slight serological relationship to the Kilham rat virus.

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EFFECT OF CHLORPROMAZINE ON CONJUGAL PLASMID TRANSFER AND SEX PILI

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The major tranquillizer chlorpromazine (Cpz) inhibited the conjugal transfer of R and F⁺lac plasmids. The frequency of transfer of R-144 and R-100 plasmids was reduced with 2–3 log by Cpz at a concentration of 50–100 µg/ml, while the frequency of RM-98 plasmid did not change under the same conditions. Cpz at 100 µg/ml was an effective inhibitor of the transfer of F⁺lac plasmid. By means of electron microscopy and plaque assay, 100 µg/ml Cpz was shown to reduce the adsorption rate of male specific ribonucleic acid phages MS-2 to the sides of F-pili. Common pili and flagellae seemed to be intact, but sex pili probably retracted in the presence of Cpz.

In our previous studies it was observed that the major tranquillizer chlorpromazine (Cpz) caused curing of R-factor and of F⁺lac plasmid from *Escherichia coli* strains [1, 2]. Recently we have found that 7,8-dioxo-chlorpromazine was an effective inhibitor of the conjugal transfer of R plasmid R-144, in spite of the fact that the drug failed to show plasmid elimination [3]. Experiments were carried out to investigate the inhibition of conjugal transfer of R-factors and that of F⁺lac plasmid in the presence of Cpz. We have attempted to elucidate whether correlation exists between the inhibition of conjugal plasmid transfer by Cpz and its effect on the sex pili of donor cells. For that purpose the effect of Cpz was studied on the adsorption of male specific MS-2 phage [4] on the sex pili of *E. coli* F⁺lac strain.

Materials and methods

Bacteria and bacteriophages. *E. coli* K12 LE 140 lac⁻ mal, Str^r carrying F⁺lac plasmid [1], *E. coli* K12 G I 65 (R 144) conferring kanamycin resistance, *E. coli* K12 G I 65 (R 100) Str^rT^rCm^rSu^r and *E. coli* K12 JE 2571 (RM 98) T^rAmp^rSm^r. These strains and MS-2 phage lysate were kindly provided by Dr. I. B. HOLLAND. *E. coli* K12 WA-1 NaN₃^rlac⁻ [5] was used as recipient strain in conjugational experiments.

Chlorpromazine-HCl (Hibernal®) EGYT Pharmaceutical Works, Budapest.

Culture media. MTY broth and MTY nutrient agar were described previously [1]. EMB agar was used for detection of lac⁺ transconjugants [1]. Oxoid Blood Agar Base broth and nutrient agar were prepared as described in "Oxoid Manual".

Inhibition of R-factor transfer. *E. coli* K12 G I 65 (R 144); *E. coli* K12 G I 65 (R 100) and *E. coli* K12 JE 2571 (RM 98) were used as donor strains and *E. coli* K12 WA-1 as recipient. From overnight precultures of donor and recipient strains dilutions were made up to the absorbance of 0.4 on the Bausch and Lomb spectrophotometer at 620 nm. Equal volumes of donor and recipient cultures were mixed and incubated without shaking in the presence

of Cpz (50 or 100 $\mu\text{g/ml}$) at 37 °C during 40 min. Dilutions were made from the mixture and 0.1 ml samples were plated on selective agar plates. Selective agar for transconjugants consisted of MTY agar, supplemented with 250 $\mu\text{g/ml}$ NaN_3 , and 50 $\mu\text{g/ml}$ kanamycin or 50 $\mu\text{g/ml}$ tetracycline. The number of donors was determined on MTY plates containing kanamycin or tetracycline, and that of the recipient on plates containing NaN_3 . Plates were incubated at 37 °C for 48 h and the colonies were counted.

Inhibition of F'lac transfer. *E. coli* K12 LE 140 (F'lac) and *E. coli* K12 WA-I strains were mixed in equal volumes as described above, and incubated in the presence of 100 $\mu\text{g/ml}$ Cpz for 40 min. The selective medium for *lac*⁺ transconjugants contained 100 $\mu\text{g/ml}$ NaN_3 in EMB agar.

Effect of Cpz on MS-2 phage adsorption. *E. coli* K12 LE 140 (F'lac) culture in stationary phase containing approximately 5×10^8 cells/ml was incubated for 1, 5 or 15 min in the presence of 50 or 100 $\mu\text{g/ml}$ Cpz at 37 °C in Blood Agar Base broth, the control culture was incubated without Cpz. Phages MS-2 were added at a final concentration $1 \times 10^6/\text{ml}$ plaque-forming units (p.f.u.), and phages were allowed to adsorb to the cells at 37 °C for 15 min. Cells and adsorbed phages were removed by centrifugation at 5000 *g* for 10 min, and the supernatant was assayed for p.f.u. of MS-2 phages by the agar overlay technique. Bottom and top agar layers included 1.5 and 0.5% agar in Blood Agar Base nutrient broth medium. *E. coli* LE 140 was used as the indicator strain. Plates were incubated at 37 °C and plaques were counted after 16 h. The direct effect of Cpz on the MS-2 phage itself was tested by treating phage lysates containing 10^6 – 10^8 p.f.u./ml with 100 $\mu\text{g/ml}$ Cpz at 37 °C for 15 min then determining the decrease of p.f.u.

Preparation of samples for electron microscopy. Of a sample of *E. coli* (F'lac) culture, about $1 \times 10^8/\text{ml}$ was mixed with 0.2 ml of phage suspension at 2×10^{11} p.f.u./ml and incubated at 37 °C for 15 min. A drop of the culture was then placed on Formvar coated carbon supported copper grid and the cells were allowed to settle on the grid for 40 min in a wet chamber containing glutaraldehyde vapour. The grids were washed twice in distilled water. Excess water was removed by filter paper, a drop of 2% uranyl acetate was placed on the grid for 10 s. The preparation was examined by a Jeol JEM electron microscope. For testing the effect of Cpz on the ability of bacteria to adsorb phages, cultures were pretreated with 100 $\mu\text{g/ml}$ Cpz for 15 min, then phages were added as described above.

Results

Effect of Cpz on the conjugal transfer of R and F'lac plasmids. Previously it has been observed that the elimination by Cpz of R-100, R 144, and RM 98 plasmids was successful up to 25, 2 and 5%, respectively (unpublished data). It can be seen from Table I, that Cpz effectively prevented the conjugal transfer of R-100 and R 144 plasmids from *E. coli* G I 65 strains to *E. coli* K12 WA-I without a significant killing of donor cells. Cpz slightly reduced the transfer frequency if the donor strain *E. coli* JE 2571 carried the RM 98 plasmid. As the Cpz eliminated most effectively the F'lac plasmid [1], we investigated the transfer of F'lac plasmid in the presence of 100 $\mu\text{g/ml}$ Cpz. The frequency of F'lac transfer proved to be higher than that of R-factors, but it decreased by 3 log in the presence of 100 $\mu\text{g/ml}$ Cpz. This concentration did not significantly influence the number of donor cells if their number was $2 \times 10^8/\text{ml}$.

Effect of Cpz on the adsorption of MS-2 phages. The decrease of *lac*⁺ transconjugants in the presence of Cpz could not be the result of F'lac elimination, considering the large size of inoculum ($2 \times 10^8/\text{ml}$). If the effect were in connection with the inhibition of mating pair formation, an alteration

Table I*Effect of chlorpromazine on the conjugal transfer of R factors and F⁺lac plasmid*

<i>E. coli</i> donor strain	Cpz μg/ml	No. of donor cells/ml	No. of trans- conjugants/ml	Frequency of transfer
K ₁₂ GI-65 (R 144)	0	2 × 10 ⁸	6 × 10 ⁶	3 × 10 ⁻²
	50	1.5 × 10 ⁸	4.5 × 10 ⁴	3 × 10 ⁻⁴
K ₁₂ GI-65 (R 100)	0	2 × 10 ⁸	5 × 10 ⁶	2.5 × 10 ⁻²
	100	1.8 × 10 ⁸	4.9 × 10 ³	2.7 × 10 ⁻⁵
K ₁₂ JE 2571 (RM-98)	0	2 × 10 ⁸	6 × 10 ⁴	3 × 10 ⁻⁴
	50	1.7 × 10 ⁸	4.9 × 10 ³	2.7 × 10 ⁻⁵
K ₁₂ LE 140 (F ⁺ lac)	0	2 × 10 ⁸	8 × 10 ⁷	4 × 10 ⁻¹
	100	1 × 10 ⁸	3.2 × 10 ⁴	3.2 × 10 ⁻⁴

Recipient: *E. coli* K₁₂ WA-I NaN₃⁺lac⁻

or lack of sex pili would occur. To check this hypothesis we measured the adsorption rate of MS-2 phage on the sex pili of F⁺lac harbouring bacteria with Cpz at the same concentration which inhibited the F⁺lac transfer. Table II shows that Cpz treated cells did not adsorb MS-2 phages as efficiently as the untreated ones. The decrease of adsorption capacity, i.e. the increase of p.f.u. in the supernatant, was in correlation with the time of Cpz treatment rather than with the concentration of the drug. If the cells were incubated in the presence of 50 or 100 μg/ml Cpz for 15 min, 30 to 40% of the total p.f.u. remained in the supernatant, in contrast to 2% in the control supernatant.

Table II*Effect of chlorpromazine on the ability of cells to adsorb male specific MS-2 phage*

Chlorpromazine		Per cent p.f.u. remaining in supernatant after 10 min adsorption*
μg/ml	time of treat- ment, min	
0	0	2
50	1	4.5
50	5	8
50	15	30
100	1	4.8
100	5	9
100	15	40

* Adsorption mixtures contained 1 × 10⁸ cells/ml incubated with 50 or 100 μg/ml Cpz for 1, 5 or 15 min, prior to the addition of 1 × 10⁶ MS-2 p.f.u./ml

The Cpz did not influence the p.f.u. of the MS-2 phage lysate (data not shown), so we suppose that Cpz has no effect on the MS-2 phage itself. This observation is in good agreement with the results of MATSUO *et al.* [6].

To answer the question whether the F-pili remain on the cell surface without adsorbing phages, or the cells have lost their pili, we examined Cpz treated cells with MS-2 phages electron microscopically. It was seen that on the cells of the control culture, the sex pili were densely covered by MS-2 phages (Figs 1 and 2), while on cells growing in the presence of Cpz there were no sex pili (Fig. 3), only common pili [7, 8] and flagella. On preparations obtained from Cpz treated cultures, no more detached pili were observed than on control preparations.

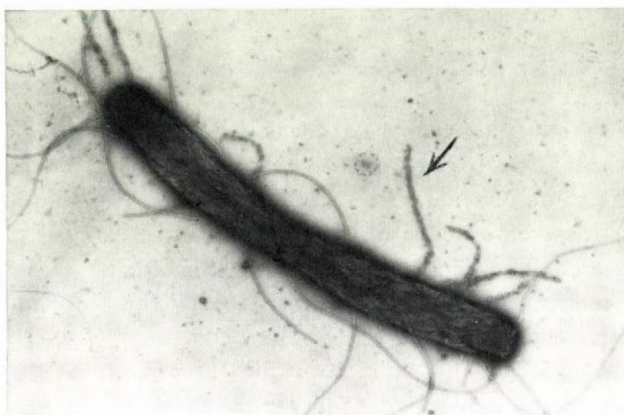


Fig. 1. Electron micrograph of a cell of *E. coli* K12 (F'lac). F-pili are identified by the attachment of MS-2 phage along their length (arrow). Magnification $\times 9000$

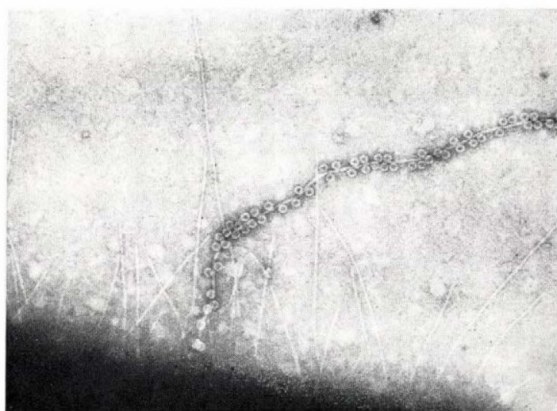


Fig. 2. Electron micrograph of *E. coli* K12 (F'lac), showing common pili surrounding cell surface; sex pili can be recognized by their longer size and by the adsorbed phages on the side. Magnification $\times 60\,000$

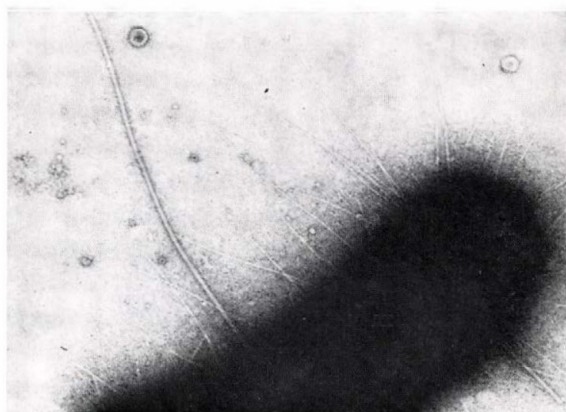


Fig. 3. Electron micrograph of *E. coli* K12 (F'lac) incubated in the presence of 100 µg/ml Cpz for 15 min before adding MS-2 phages. Only common pili and flagella are seen. Magnification $\times 60\,000$

Discussion

Chlorpromazine has numerous effects on the properties of surface membranes of higher organisms [9–11] and, as reported recently, also of bacteria [12]. Earlier we have suggested that plasmid curing by psychoactive drugs may be due to their action on the bacterial surface, perhaps leading to a failure of plasmid molecules to segregate daughter cells [1, 3]. In the present experiments, the inhibition of conjugal plasmid transfer may have arisen through the inhibition of initial mating pair formation rather than by a direct effect on plasmid DNA. There are data on the interaction of Cpz with DNA [13–16], but we have no data that Cpz would pass through the cell membrane. On the other hand, a *de novo* conjugal DNA synthesis of the donor is not obligatory for the transfer of plasmid DNA [17, 18]. It was therefore reasonable to suppose that the inhibition of mating pair formation might be due to the effect of Cpz on the sex pili. The role of sex pili in conjugation has recently become more elucidated. They are essential on donor cells for the initial step of conjugation and are not needed for DNA transfer if more stable contacts have formed [19]. Since MS-2 receptors are located on the sex pili [8], it is assumed that not only the conjugal plasmid transfer but adsorption of phages to pili is also inhibited by growth of the cells in the presence of Cpz. The experiments described in Table II show that this was actually the case. The p.f.u. of non-adsorbed phages increased twentyfold in the presence of Cpz. By electron microscopy there were no sex pili with adsorbed phages on their sides; they seemed to have disappeared from the cell surface. The cause of this could not be a detachment of the pili, because no trace

of it was detected by electron microscopy. The disappearance of sex pili may have been due to their retraction, of which there is some evidence [20-22], especially under the effect of NaCN or low temperature [23]. FOLKHARDT *et al.* [24] suggested that retraction of the pili occurs on a conformational transition from ordered helix to sheet structure of subunits, which could then collapse and possibly be absorbed into the cell envelope. Retracted pili might be short enough to escape detection by electron microscopy.

Flagella and common pili were not affected by Cpz. It is known that both of them have a structure different from that of the sex pili, but the selective effect of Cpz on them is not understood. As the pili consist of protein [24, 25], Cpz might bind to them, or to the point of outgrowth of pili. BAYER [26] found that F pili are anchored at adhesive sites between the inner and outer membranes, so we suppose that this area could be one of the target sites of Cpz.

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INDEX

<i>Gupta, J. K., Shirkot, C. K., Dhawan, S.</i> : Growth of Bacteria and Yeast on Enzymically Degraded Alkali Treated Rice and Wheat Straws	131
<i>Bakalirarov, D., Kostov, O.</i> : Soil Microbiological Assessment of Toxicity of Alkanoate, Amide and Other Herbicides	141
<i>Ádám, É., Nász, I.</i> : Connections between the Hexon Polypeptides in the Two-Dimensional Hexon Crystalline Array	147
<i>Gönczöl, É., Boldogh, I., Vácsi, L.</i> : IgG-Fc-Binding Receptors in Cells Abortively Infected, or Transformed, by Human Cytomegalovirus	157
<i>Kramer, M., Kecskés, É., Horváth, I.</i> : Guanosine Polyphosphate Production of <i>Escherichia coli</i> Stringent and Relaxed Strains in the Stationary Phase of Growth	165
<i>Milch, H., Nguyen, T. K.</i> : <i>Klebsiella</i> and <i>Enterobacter</i> Strains Derived from Hospital Infections. II. Occurrence and Characterization of R-, Lac- and Col-Plasmids and their Clinical-Epidemiological Significance	171
<i>Tóth, M., Lengyel, A., Béládi, I., Nász, I.</i> : Characterization of a Parvovirus Strain Isolated From Human Adenovirus Type 12	197
<i>Mándi, Y., Molnár, J.</i> : Effect of Chlorpromazine on Conjugal Plasmid Transfer and Sex Pili	205

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R-PLASMID STUDY OF AN OUTBREAK CAUSED BY MULTIRESISTANT STRAINS OF *SALMONELLA PANAMA*

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(Received April 8, 1980)

A *Salmonella panama* outbreak was observed in the months May through July, 1979, in the newborn and intensive wards of a paediatric hospital. The isolates proved to be multi-resistant to antibiotics. To clarify the presumed R-plasmid nature of the multiresistance, the transfer of the resistance to five antibiotics, viz. ampicillin, chloramphenicol, streptomycin, kanamycin and tetracycline, was studied. *Escherichia coli* K12 NaI^r, the strain used as recipient, acquired resistance simultaneously to four antibiotics, viz. ampicillin, chloramphenicol, streptomycin and kanamycin. The minimum inhibitory concentration for the transconjugants agreed well with that of the original *S. panama* strain. Resistant and multiresistant *E. coli* strains not belonging to serogroups associated with infantile enteritis were isolated from the intestinal flora of several patients and symptomless carriers. Elimination of the transmissible multi-resistance was observed in 0.15–1.1% of the strains.

Recently, salmonellosis outbreaks including serious cases in humans have occurred all over the world. It was observed in several countries that only few widely spread were *S. typhi-murium*, *S. enteritidis* and *S. london*. *S. panama* is the most frequently occurring serotype in West Berlin, and it is common elsewhere in Europe [2]. The salmonellosis outbreaks observed in Hungary in the years 1972–1976 were reviewed by RUDNAI *et al.* [3]. Among the 18 serotypes commonly found in the years 1972–1974, three disappeared in the following two years, whereas three types occurred only in the last two years of the five-year period. In Csongrád county, like elsewhere in Hungary, *S. typhi-murium* was found most frequently, followed in rank by *S. london*. *S. panama* was the eighth in sequence. Taking into account the type distribution of the hospital outbreaks that spread by contact in the five-year period in Hungary, *S. panama* was the second in frequency. Six outbreaks including 173 cases were caused by this serotype.

Since the early 'seventies, multiresistant strains carrying R-plasmids have become more and more frequent among the strains causing salmonellosis outbreaks [4–9]. The R-plasmid of multiresistant *S. wien* strains was analysed by McCONNELL *et al.* [10]. The first outbreak caused by this strains was observed in Algeria in 1969, and this was followed by outbreaks in Southern and Western Europe, mainly in hospitals and paediatric departments. Most of the isolates were multiresistant and carried the same transferable R-plasmid.

The geographic distribution of the R-plasmids responsible for salmonella multiresistance was mapped by ANDERSON [11]. This author found a clone-

like selection of salmonellae, first of all *S. typhi* and *S. typhi-murium*, in some large territories, e.g. the Middle East, South America and Northwestern Europe. Sometimes the R-plasmid, presumably originating in a single clone, was confined to a single hospital. The selection and the prolonged dominance of an organism carrying R-plasmid may be influenced by environmental factors as well.

The resistance to antibiotics of the *S. typhi-murium* strains isolated in Hungary in the years 1971–1973 was examined by MILCH *et al.* [12]. Resistance to one or more antibiotics was observed in 4.5% of the isolates, and 80.5% of the resistant isolates were carrying transferable R-plasmids.

In the present work, multiresistant *S. panama* strains associated with an outbreak in a preterm baby's ward were examined. The purpose was to determine the resistance spectra, the resistance levels of isolates and the transferability of the resistance, and to investigate transfer by genetic methods.

Materials and methods

Strains. Ten *S. panama* and six *E. coli* strains were studied. They were isolated from subjects affected by the outbreak at different times. The strain *E. coli* K12 Hfr $\text{NaI}^+ \text{lac}^-$ was used as recipient.

Culture medium. Composition of the broth medium: Bacto peptone (Difco), 1 g; Lab-Lemco meat extract (Oxoid), 10 g; NaCl, 6 g; distilled water ad 1000 ml; pH 7.4. The broth was autoclaved at 121 °C for 30 min. The solid medium contained 11 g Oxoid agar in 1000 ml broth. Antibiotic resistant transconjugates were selected on eosin–methylene blue agar medium. The medium was completed by adding chloramphenicol (25 µg/ml) or ampicillin (25 µg/ml), or streptomycin, kanamycin or tetracycline (each 50 µg/ml) and, in every case nalidixic acid (50 µg/ml).

Measurement of resistance to antibiotics. Resistest® disks (Institute for Serobacterial Production and Research Human, Budapest) were used according to the manufacturer's prescription. The following disks were applied: ampicillin, chloramphenicol, streptomycin, kanamycin, tetracycline, carbenicillin, neomycin, co-trimoxazole, nitrofurantoin and nalidixic acid.

Determination of the minimum inhibitory concentration (MIC). The serial dilution method was performed in test tubes, using 10^5 cells as inoculum. The tubes were incubated at 37 °C for 48 h and the bacterial growth was observed in the presence of 25–1000 µg/ml of the antibiotic drug.

Transfer of R-plasmids. The strains to be used as donor and recipient were cultivated in nutrient broth at 37 °C. From logarithmic phase cultures 0.1 ml or 0.2 ml volumes were measured into 5 ml broth, and the tubes were incubated at 37 °C for 18 h. Then the broth culture was spread on selective media. In each case, 10 transconjugate colonies were subjected to a second plating and studied for transferred resistance. The frequency of transmission was related to the recipient strain.

Phage typing. A standard series of *E. coli* phages [13] was used in the phage typing of transconjugant *E. coli* strains and *E. coli* isolates.

The male character of R-plasmids was determined with the male-specific RNA phages MS2 and ϕ_2 .

Deletion of the resistance to antibiotics was checked after an incubation in broth containing 10 µg/ml chlorpromazine for 24–48 h [14]; spontaneous loss of resistance was examined after 6 weeks incubation at room temperature. About 100 isolated colonies were spread on plates containing antibiotic and the colonies that had lost their resistance were tested after two consecutive platings by the disk method.

Results

In the wards under study, *S. panama* was isolated from the faeces on nine infants. The first strain was isolated early in April, followed by further isolations from mid-May until July 31, 1979. Seventy-three strains were isolated altogether, 2-17 from each positive child. The duration of shedding ranged between 4 days and 8 weeks (Table I).

From three children, *E. coli* was isolated beside *S. panama*. In one of these, three consecutive isolates showed a broadening of the resistance spectrum. The first isolate was resistant to ampicillin and chloramphenicol, the second to the same and streptomycin, and the third to kanamycin and tetracycline. In the second case, *E. coli* and *S. panama* of identical resistance spectrum were isolated from a single faecal sample. From the third child, *E. coli* strains resistant to ampicillin and chloramphenicol were isolated on two occasions. These isolates, though they did not belong to serogroups associated with infantile enteritis, showed an identical phage picture, viz. phage patterns 9, 16, 17, 18; phage group A₃B₂. The distribution of the *S. panama* and *E. coli* isolates by resistance to antibiotics is shown in Table II.

Of the *Salmonella* strains isolated from each patient, one, occasionally two, altogether 10, were examined in detail. The resistance spectrum was determined by studying their resistance to 10 antibiotics. All isolates proved to be resistant to ampicillin, chloramphenicol, streptomycin, carbenicillin and nitrofurantoin and all but one to kanamycin.

The resistance of *Salmonella* strains to nalidixic acid is advantageous in the selection of transconjugants originating from conjugation with the *E.*

Table I
Period of excretion of *S. panama*

Patient	Onset	Duration	Distribution of 73 isolates
	of excretion, days		
T. Cs.	April 10	54	9
Cs. Zs.	May 16	64	15
R. H.	May 18	4	2
M. H.	May 23	35	7
F. A.	May 28	4	2
Sz. A. +	June 5	56	17
Sz. F. +	June 11	50	14
N. A. +	June 12	7	2
D. J.	June 21	28	5

+ Simultaneous isolation of *E. coli*

Table II
Distribution of the resistance of the isolates

Bacterium	No. of isolates tested	Resistance	ampicillin	chloramphenicol	streptomycin	tetracycline	kanamycin	carbenicillin	neomycin	nitrofurantoin	co-trimoxazole	nalidixic acid
<i>S. panama</i>	10	resistant	10	10	10		9	10		10		
		sensitive				10	1		10		10	10
<i>E. coli</i>	6	resistant	6	6	3	1	2	6			6	
		sensitive			3	5	4		6	6		6

coli K12 NaI^r strain. The transfer of resistance and the MIC value were examined with five antibiotics, *viz.* ampicillin, chloramphenicol, streptomycin, kanamycin and tetracycline. Simultaneous transfer of resistance was observed for four antibiotic drugs, *viz.* all but tetracycline. The frequency of transmission ranged between 10^{-4} and 4×10^{-4} . The MIC values for the conjugates were equal to, or by an order of magnitude lower than, the values for the *Salmonella* strains. Table III shows data for a few characteristic strains. All strains except No. 530, which was sensitive to kanamycin, behaved identically with strain No. 256. The multiresistant *E. coli* strains isolated simultaneously are represented in Table III by strain No. 349. This strain was somewhat different to tetracycline and co-trimoxazole and sensitive to nitrofurantoin. The resistance to chloramphenicol was not transferred from this strain to the recipient. Both resistances were transferable with similar frequencies from *E. coli* isolates resistant to ampicillin and chloramphenicol. The MIC value was identical with the value obtained for the donor strain in the case of ampicillin, it was lower, even by one order of magnitude, than the other three antibiotic drugs (Table III).

The transconjugant *E. coli* strains did not differ from the original *E. coli* K12 in phage picture. All the examined plasmids were of *fi*⁻ character.

The prototype strains were examined for spontaneous loss of resistance and its elimination by chlorpromazine. Spontaneous loss was observed in 0.15%, elimination was successful in 1.1%. Although the transfer of resistance against four antibiotics occurred simultaneously, the *Salmonella* strains lost their resistances gradually. The loss of resistance to kanamycin was the most frequent, followed by the joint loss of resistance to kanamycin and streptomycin. Among more than 1000 colonies, a single one proved to be sensitive to chloramphenicol and none of the colonies were resistant to ampicillin. Presumably, the loci responsible for the last two resistances are closely linked in the plasmid.

Table III

Antibiotic resistance values for isolates and transconjugants

Strain			ampicillin	chloramphenicol	streptomycin	kanamycin	tetracycline	carbenicillin	neomycin	co-trimoxazole	nitrofurantoin	nalidixic acid	Minimum inhibitory concentration (MIC) $\mu\text{g/ml}$				
<i>S. panama</i>	<i>E. coli</i>	transconjugant											ampicillin	chloramphenicol	streptomycin	kanamycin	tetracycline
No. 256			R	R	R	R		R	R	S	R	S	>1000	>1000	>500	>1000	>25
		K12/256	R	R	R	R	S	R	S	S	S	R	>1000	>500	>250	>500	
No. 530			R	R	R	S	S	R	S	S	R	S	>1000	>1000	>500		
		K12/530	R	R	R	S	S	R	S	S	S	R	>1000	>1000	>250		
	No. 349		R	R	R	R	R	R		R	S	S	>1000	>1000	>1000	>1000	>1000
		K12/349	R	R	R	R	R	R		R	S	R	>1000		>250	>1000	>1000
	No. 347		R	R	S	S	S	R	S	R	S	S	>1000	>250			
		K12/347	R	R	S	S	S	R	S	S	S	R	>1000	>100			

R = resistant, S = sensitive

Discussion

CHAU *et al.* [9], who isolated *S. johannesburg* strains between 1973 and 1977, were the first to call attention to the joint appearance and joint transferability of resistance to ampicillin and chloramphenicol. They found the level of chloramphenicol resistance between 320 and 640 $\mu\text{g/ml}$, but the resistance transferred to the recipient strain was only about one quarter of the level.

Based on the present results we assumed that the *S. panama* strains isolated by us, or the plasmids carried by the strains, were of common origin. This view was supported by the identical resistance spectrum and the identical or nearly identical MIC values.

It was recognized early during the outbreak that the resistance was plasmid-mediated. Therefore, in addition to *Salmonella*, we sought for *E. coli* strains in the faecal samples to show whether resistance had been transferred *in vivo*. Of the several *E. coli* isolates, two proved to be multiresistant. One agreed with the epidemic strain in antibiogram, the other carried additional resistance to tetracycline. The plasmid carried by strain No. 349 (Table III) was very similar in resistance determinants to the R-plasmid of the multiresistant *S. london* strain that had been isolated from an outbreak in Csongrád county in the years 1976–1978 [15]. The outbreak had originated from meat products and, like in the outbreak under study, the multiresistant strains were selected in a hospital milieu.

Although the newborn babies yielding the isolates had not been treated with antibiotics before faecal samplings, the hospital milieu was favourable for the selection of multiresistant strains. The source of infection could not be identified.

It is not known whether an R-plasmid of an *E. coli* had originally been transferred to the *S. panama* strain, or whether the R-plasmid responsible for the multiresistance had been transferred to *E. coli* strains in the intestinal flora of patients.

The invasiveness and colonization capacity of a *Salmonella* are well-known to play an important role in spreading of the organism. Outbreaks may continue spreading even after the focus of infection has been eliminated [16]. This is because persistent implantation and circulation of the organism in symptomless carriers may lead to cross infections in hospital. ANDERSON [11] suggests that the appearance in a propitious moment of a single microorganism carrying a certain plasmid may result in an outbreak with unpredictable consequences. The increasing genetic transfer is expected to be accompanied by an increase in the number of recombinations. Thus, bacteria carrying both R-plasmid and virulence factor may arise.

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MUTUAL ORIENTATION OF ADENOVIRUS HEXON POLYPEPTIDES IN A TWO-DIMENSIONAL CRYSTALLINE ARRAY

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Three profiles of hexons were detected in two-dimensional adenovirus crystalline arrays: (i) approximately ringwise closed hexons with a roundish hole in their centres and with 2–4 electron dense spots in the wall of the rings; (ii) hexons consisting of three approximately oblong polypeptides enclosing a triangular hole; (iii) triangular hexons, containing three main polypeptides with an Y-shaped slit, instead of a hole, in their centres. Following the examination of directly or computer corrected electron micrographs a tentative model has been developed on the possible mutual "rotational" orientation of hexon polypeptides within the two-dimensional crystalline array. The position of hexons is such that the longer side of a polypeptide of each hexon is next to the end of two polypeptides of its neighbouring hexon, i.e. one polypeptide is linked to two other ones. An irregularity evolves in this "one-to-two" linkage system by the rotation of a hexon, the maximum rotation being 60°, as follows from the hexon's threefold symmetry. The presence of lying hexons and of hexons of different contours, points to irregularities in "vertical" orientation, with the turn reaching even 90° or 180°, i.e. the hexons might be connected in such a way that their originally external or internal parts are facing identical directions.

Adenovirus is a medium-sized, DNA containing virus of regular icosahedral shape. Its protein coat is composed of 252 morphological units, capsomers [1, 2]; 12 of these are situated at the 12 vertices of the icosahedron and these capsomers, together with their adjoining projections (fibres), constitute the structural elements called penton. The 240 capsomeres, which are arranged on the faces and edges of the icosahedron, are called hexons [3]. Data on the ultrastructure of the hexon capsomers, obtained mainly for serotypes 2 and 5, are not completely concordant regarding to the shape and size of hexons [4–8]. Nevertheless, it can be taken for certain that a hexon consists of three polypeptide subunits and, accordingly, hexon capsomeres have a threefold symmetry axis [7, 9–11]. Highly purified hexon capsomers can be crystallized by a specific procedure [12, 13] which yields three-dimensional tetrahedral crystals as well as tightly packed two-dimensional crystalline arrays [12–14]. The hexons of the latter array form a hexagonal packing similar to the hexon capsomers in the capsid of virions. Research on the mode of linkage and the orientation of capsomers in the capsid is of great importance and the two-dimensional crystalline arrays with their great number of periodically linked hexons, can be used as a suitable model.

Materials and methods

Virus and cell culture. The prototype strain of adenovirus type 1 was studied. It was propagated on HEp-2 cell culture in one and two-litre Roux flasks. The cell culture was maintained in Parker's 199 medium containing 15% calf serum and the usual antibiotics. Following infection, Parker's 199 medium supplemented with 3% rabbit serum was used as a maintenance medium.

Production and crystallization of hexons. After total cell degeneration, the cells were separated from the maintenance medium by centrifugation at 2000 rpm. The infected cells were destroyed either in a homogenizer or in an ultrasonic disintegrator.

(i) **Ultracentrifugation.** Separation of virions from the soluble components was performed on a double CsCl cushion in a 3×20 ml swing out rotor by centrifugation at 27 000 rpm for 90 min.

(ii) **Anion exchange chromatography.** Separation of the soluble protein components from above the virion band in the ultracentrifuge tube was performed on a DEAE Sephadex A-50 anion exchange column. The eluent used was 0.01 mole/l phosphate buffer with increasing NaCl concentration. The hexon-containing fractions were further purified by repeated anion exchange chromatography.

(iii) **Gel filtration.** The hexon preparations, separated and purified by anion exchange chromatography, were further purified by gel filtration on a Sephadex G-200 column. The eluent was 0.01 mole/l phosphate buffer pH 7, with 0.15 or 0.5 mole/l NaCl, containing 2×10^{-4} NaN_3 in order to prevent bacterial contamination. Fractions were collected by an LKB Ultrac device with an Uvicord II absorption photometer.

(iv) **Protein concentration** was estimated according to LOWRY *et al.* [15].

(v) **Immune sera.** In order to identify the virus proteins of the fractions, immune sera were prepared by immunizing rabbits against different soluble protein components of adenovirus in Freund's complete adjuvant [16].

(vi) **Electrophoresis.** The homogeneity of the hexon preparations was determined by polyacrylamide-gel electrophoresis, performed in a gel containing 50–70 g/l acrylamide and 1.8 g/l $\text{N}'\text{N}'$ -bismethylene acrylamide. Polymerization was performed by using 2.3 g/l tetramethyl-ethylenediamine and 1.4 g/l ammonium persulphate or UV light irradiation. Electrophoresis was carried out at 2–4 mA/column for 1–3 h. A tris-glycine buffer (H-ion concentration 5 nmoles/l = pH 8.3) was used as electrolyte. The gels were stained with amidoschwarz and then estimated visually or by the Kipp-Zonen densitometer.

(vii) **Crystallization.** The purified and concentrated hexon preparations, proved homogeneous by polyacrylamide-gel electrophoresis, were dialysed against a 0.5 mole/l acetate buffer (H-ion concentration 32 $\mu\text{moles/l}$ = pH 4.5) at $+4^\circ\text{C}$ for three days and subsequently stored in test tubes at $+4^\circ\text{C}$. The procedure led to the development of three-dimensional tetrahedral crystals and, in addition, monolayer hexon crystalline arrays, as observed under the electron microscope [12, 13].

Freeze-fracturing. The test materials were frozen in two flat fitted gold disks, in Freon 22 and then in liquid nitrogen. Subsequently, the frozen preparations were broken up with a knife. The broken surfaces were steamed, first with platinum at an angle of 45° then by carbon at a right angle. The replica thus obtained shows the relief of the original surface and can be studied by electron microscopy.

Electron microscopy. After dialysis against acetate buffer, the samples were adsorbed to carbon-formvar coated grids, stained negatively with 1% uranyl acetate. A JEM 100 B electron microscope was used at 60 kV. The electron microscopic negative films were enlarged to a total magnification of 1 : 300 000 or 700 000. The disturbing background granulation was diminished by underfocussing [6, 17].

Computer processing of electron micrographs. Slides made of the electron microscopic negative films were projected and an arbitrarily chosen part was digitized by an electronic image-digitizer with a TV camera with Zoom objective. The equipment is essentially a special computer for scanning the selected image sector into 8064 points (72 rows with 112 image points each) and digitizing them on a scale of eight grades of grey spectrum. Thus, every point was characterized by a digit from 0 to 7. The range could be regulated according to the required precision of colour resolution, i.e. 0 might correspond to black and 7 to white or, 0 to a darker grey and 7 to a lighter grey. The digits, describing the image points, i.e. the digitized image, were punched on tape and then processed in a R-20 type computer (Computer Centre, Semmelweis University Medical School). Data processing was done by printing the digits corresponding to the image points, with the exception of those standing for certain

grades, e.g. dropping the values that describe the grades 0–3, only the grades 4 and 7 were printed, cp. Fig. 9. Thus, details below or above a certain grade of grey were left out, in order to sharpen other details.

Results

Shape and intrahexonal location of the hexon polypeptides in the crystalline array. A hexon consists of three polypeptide subunits and, accordingly, a hexon has a threefold symmetry axis [5, 7, 10, 18, 19]. In negatively stained preparations the three morphological subunits, i.e. the three main polypeptides, are seldom clearly discernible. This is due either to a positional shift or bend of one end of each polypeptide or to the procedure of negative staining. Thus, hexons might seem to consist of more than three subunits [7]. Analysing numerous two-dimensional crystalline arrays we found that the bulk of the hexons display ringwise shapes with an approximately round hole in their centre. Nevertheless, 2–4 electron dense spots could be observed in the ring-wall, especially on underfocussed high resolution pictures (Fig. 1). In this case the contours of the single polypeptides were blurred. On the other hand, there were two hexon types where the number and shape of the polypeptides they consisted of, were clearly discernible. The three main polypeptides constituting the hexon of the first type were either like round cornered parallelepipeds or slightly bent parallelepipeds. These oblong polypeptides were linked by their thinner sides and thus the threefold symmetry and the approximately triangular hole enclosed by the three polypeptides were clearly visible (Fig. 2). The linked polypeptides form a triangle in this type of hexon. Since the junctures of the polypeptides are approximately as large as the longer sides of the polypeptides, these hexons are approximately hexagonal. This hexon was frequently seen in the two-dimensional crystalline arrays. The other hexon type showed an even more expressed triangular

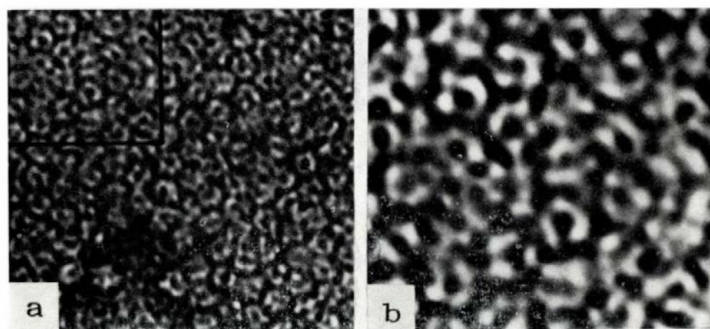


Fig. 1. (a) Electron micrograph of part of a two-dimensional hexon crystalline array. Stained by uranyl acetate, $\times 300\,000$. (b) An enlarged part of the same crystalline array, underfocussed, $\times 700\,000$. The hexons are mostly closed with a roundish hole in their centre, and there are 2–4 electron dense spots in the wall of the ring

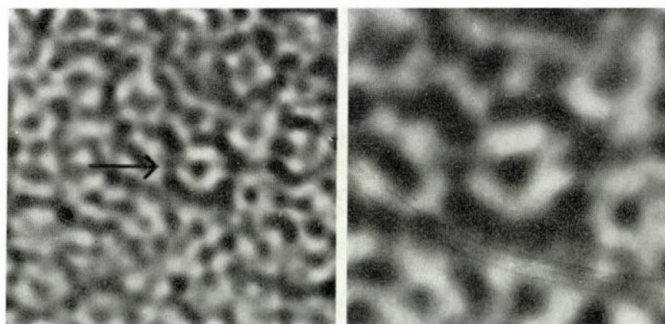


Fig. 2. The arrow points to a hexon in the picture to the right which clearly shows the approximately oblong profile of the three polypeptides and the triangular area they enclose. The size of the juncture of two polypeptides approximate that of the longer side of a polypeptide. Thus, in spite of its clearly visible threefold symmetry, such a hexon is in an approximately hexagonal area

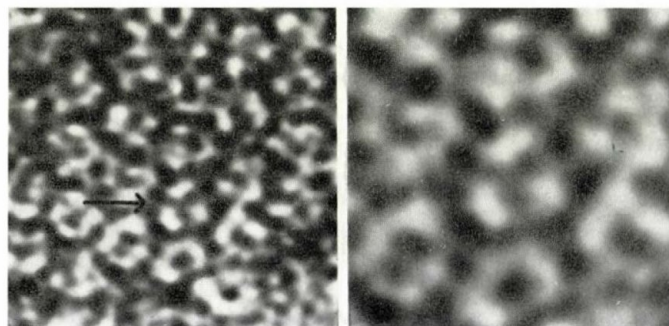


Fig. 3. Hexon forming an almost equilateral triangle (arrow). The three main polypeptides the Y-shaped slit between them, and the threefold symmetry are seen. The profile of the polypeptides are approximately rhomboidal. Enlarged to 1 400 000 in the picture to the right

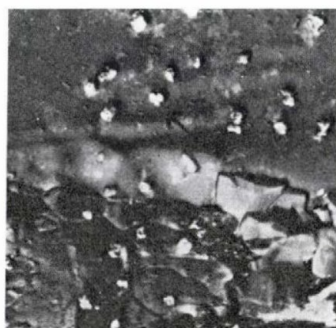


Fig. 4. Electron micrograph of a freeze-fractured homogeneous hexon preparation. The arrow points to a hexon that clearly reveals the triangular shape, the three main components and the Y-slit between them

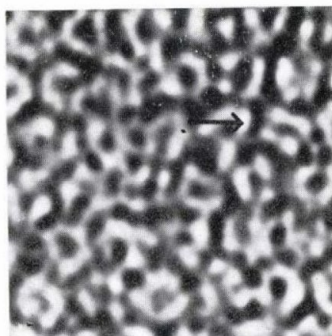


Fig. 5. "Lying" hexon with only two visible polypeptides and the slit between them

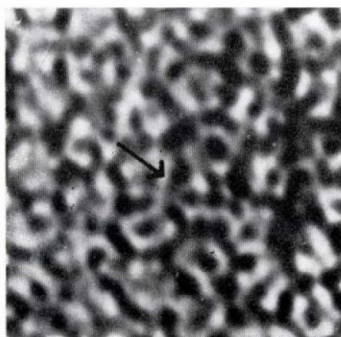


Fig. 6. The arrow points to a hexon with two holes

shape and clearly consisted of three main polypeptides. Nevertheless, the area enclosed by its six nearest neighbours, in which it is located, is hexagonal. This type was much less frequent than the former one. Its shape was approximately an equilateral triangle, while the slit in its centre was not round but narrow, Y-shaped. The profile of its polypeptides was approximately rhomboidal (Fig. 3). Such hexons were observed in electron micrographs of freeze-fractured hexons, too (Fig. 4). According to NERMUT and PERKINS' studies [11] this hexon form is characteristic of external hexon ends (hexon tops) located at the surface of virions. Hexons situated horizontally, i.e. lying hexons could rarely be found in the array. They showed just two polypeptides with a longitudinal slit between them (Fig. 5). Hexons that seemed to have two central holes (Fig. 6), occurred as well. There were in addition hexons in which the three polypeptides did not form a triangle but showed a decomposed, loosening, diverging structure (Fig. 7). The net of the interhexonal connections interlacing the whole crystalline array (Fig. 8) generally makes it extremely difficult to estimate the shape and arrangement of hexons and polypeptides [19].

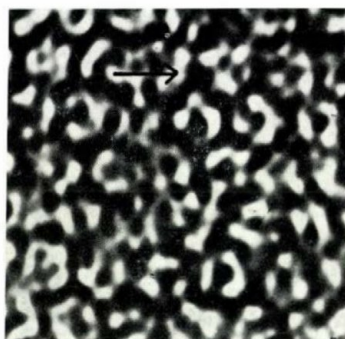


Fig. 7. The arrow points to a hexon with clearly discernible three main polypeptides, which, however, show a diverging pattern

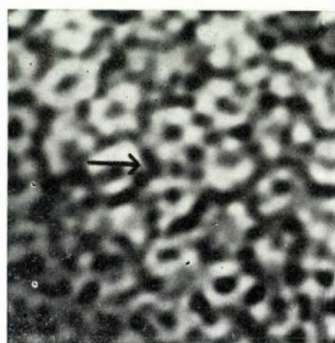


Fig. 8. The interhexonal net of connective elements in a two-dimensional crystalline array (arrow)

Mutual orientation of hexons and polypeptides within the crystalline array.

Computer processing of the electron micrographs revealed three highly electron dense spots in a hexon. This is a further evidence that a hexon is built up of three polypeptide subunits, and that the ends of these subunits converge, forming a triangular shape. It can be estimated that the polypeptides of two neighbouring hexons in the crystalline array are situated so that one polypeptide of a hexon is nearest to two polypeptides of the neighbouring hexon, i.e. a polypeptide is always linked to two other ones. Thus, the hexon polypeptides are linked according to a "one-to-two" pattern in the crystalline array, similarly to the linkage within a complete hexon, with the difference that the polypeptides converge in the hexon (Fig. 9). When the triangular shape of neighbouring hexons and the three main polypeptides are distinguishable in electron micrographs, the longer side of a hexon polypeptide is seen to be connected with the ends of two polypeptides of the neighbouring hexon (Fig. 10). This "one-to-two" type connections between the polypeptides of neighbouring hexons were found, after computer image-correction, between

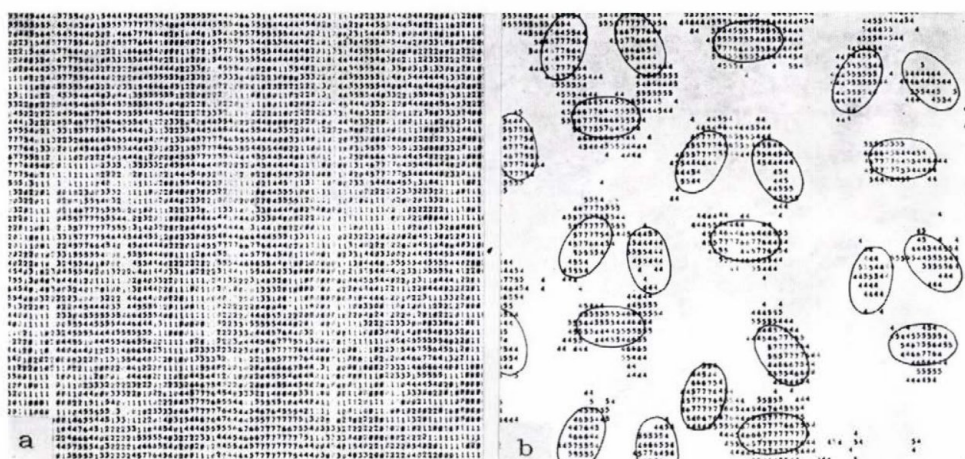


Fig. 9. (a) Digitized electron micrograph of a two-dimensional crystalline array. (b) Mutual orientation of hexon polypeptides as seen after computer image-correction. The "one-to-two" orientation of polypeptides within and between the hexons are well visible

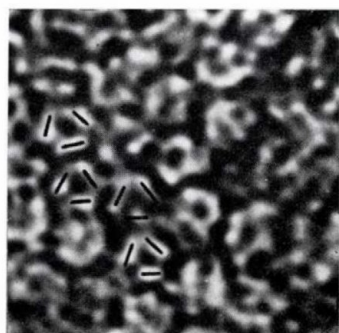


Fig. 10. "One-to-two" linkage of polypeptides. Electron micrograph. The longer side of a polypeptide is next to the ends of two polypeptides of the neighbouring hexon (four hexons are inked in)

the afore-mentioned hexons with two different types of contour (Figs 2, 3) as well (Fig. 11).

We drew up a model of the possible mutual orientations of hexon polypeptides in the two-dimensional crystalline array on the basis of direct electron microscopic observations and by computer image correction (Fig. 12). The hexons are in this "one to two" arrangement, orientated identically as regards the position of the polypeptides, and have an equivalent environment, corresponding to the hexagonal packing and threefold symmetry axis. This arrangement might be the rule for two-dimensional crystalline arrays, at least for distinct parts of it. Irregularities occur if some hexons rotate a few degrees from the position of the rest. It is in the nature of these structures that the

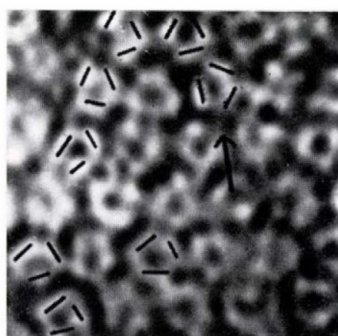


Fig. 14. Electron micrograph of a two-dimensional hexon crystalline array. The estimated orientation of hexon polypeptides is seen. The arrow points to a hexon with an approximately 60° rotational deviation from the general orientation of polypeptides

greatest deviation is caused by a rotation of 60° when "one-to-one" or "two-to-two" linkages ensue between the neighbouring hexons (Fig. 13). A further rotation means a gradual return to the original position, since a further rotation of 60° restitutes the original orientation, as follows from the threefold symmetry axis. Such irregularities may be seen on the electron micrographs as well (Fig. 14).

Discussion

Electron microscopic studies on virus structure are increasingly furnishing quantitative data and admit functional conclusions, especially since the introduction of new methods of electron micrograph analysis such as optical diffraction or computer analysis and modelling. The success of image-analyses depends on the regular incidence of repeating conformations in large numbers on the original electron micrograph. It is difficult to obtain natural biological structures with highly organized complex structures and sufficient periodic details that can clearly be distinguished from the background noise of the electron microscopic image. Analysis of electron microscopic images of crystals or paracrystalline arrays, obtained *in vitro* from biological particles in which the periodical information is distributed over a large area, may surmount this difficulty [20]. Thus, a two-dimensional adenovirus hexon crystalline array is especially suited for studying the mutual orientation of hexon polypeptides.

Our tentative model on the orientation of hexon polypeptides expresses the interrelation of threefold symmetry and hexagonal packing for the three polypeptides that form a complete hexon. The "one-to-two" orientation represents the "rotational" orientation in the plane of the picture. This orientation may be the general rule in the two-dimensional crystalline array. Examining a wide area, clear judgement is affected by numerous factors, like a shift or

bend of a polypeptide-end in relation to its other end; the nature of negative staining; a 90° or 180° deviation of some hexons from the vertical axis; the network of inter- and intrahexonal connections between the hexon polypeptides, as it is often impossible to differentiate the hexon polypeptides from connecting elements [19]; the short and long range disorder of the hexagonal packing; additional hexon rows or dislocations [14]; a rotation of hexons in relation to their neighbours; or the rotation apart of larger crystalline array parts from one another.

Considering the fact that we could detect two different types of hexon with distinct profiles in the crystalline array which might be equivalent to the external and internal ends of hexons (Figs 2, 3), irregularities in the "vertical" orientation may also occur. That means the presence in the picture plane of both the hexon end-parts facing the external surface and of those facing its internal part in the capsid of the virion. According to NERMUT and PERKIN's hexon model, there is not a hole but a Y-shaped slit at the external end of the hexon. This type of hexon-end was infrequent in the crystalline arrays examined by us. Thus, we assume that it was the profiles of the inner hexon ends near the core that we saw in these crystalline arrays.

Acknowledgement. We are indebted to Dr. L. FEDINA for computer image analyses.

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THE COLONIAL MICROTTEXTURE OF HUMAN MYCOBACTERIAL STRAINS ISOLATED IN BURMA*

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Sixteen strains of *Mycobacterium tuberculosis* isolated in Burma were investigated for microcolonial texture using the thin section technique developed at the WHO Collaborating Centre in Prague. The strains were grown in deep (Šula's liquid medium) and surface cultures (Loevenstein-Jensen medium). Their colonies killed by 10% formol were embedded in 2% agar and paraffin, cut by a Reichert microtome and stained by the Ziehl-Neelsen technique. According to the surface texture and deep growth pattern, the Burmese strains were divided in three different groups. The European type was characterized by a growth strictly confined to the surface of the Loevenstein-Jensen medium, the African type by combined surface and subsurface growth, and the Burmese type not seen in Europe and Africa by non acid-fast granular and acid-fast colonies with distinct formation of strongly acid-fast cords both in surface and subsurface growth.

Studying the colonial microtexture of mycobacteria is a technique not widely used because of the difficulty of preparing thin sections from such a fragile material like mycobacterial colonies grown on solid or in liquid media. This technique, however, is rewarding as it allows to examine the details of the inner structure of mycobacterial colonies and their subcolonial growth features important for taxonomic studies of mycobacteria.

In this way it was possible to differentiate three different types of Burmese mycobacteria, some of them resembling *Mycobacterium bovis* or *Mycobacterium africanum*.

In previous studies we reported some results obtained in human and bovine mycobacteria isolated in Czechoslovakia and Argentina, investigated in thin sections of colonies grown on Loewenstein-Jensen egg (L-J) or Šula's semisynthetic liquid medium. The most peculiar finding was the demonstration of phage colonial microlysis observed in Argentinian bovine strains and the subcolonial growth in the subsurface layers of the L-J medium never seen in human mycobacteria isolated in Czechoslovakia or other European countries. These two newly discovered phenomena may therefore be used as additional markers for the differentiation mycobacterial taxons [1, 2].

During my stay in Burma in the capacity of WHO consultant in tuberculosis in 1978, I encountered in Rangoon and Mandalay in a certain number of sputum smears stained by Ziehl-Neelsen technique "tadpole" forms of

* This study was supported by a grant from the World Health Organization

acid-fast rods never seen in Europe or Latin America. These mycobacteria were slightly curved rods, very thin on one end, while the other end was enlarged, containing a large strongly acid-fast granule, resembling the typical clubs of the diphtheria bacillus. As these extraordinary forms of acid-fast microorganisms have never been observed in our European and extra-European pathological specimens examined for mycobacteria at the WHO Collaborating Centre for Mycobacteria, additional examinations including the technique of thin colonial sections were undertaken in Prague.

Materials and methods

Twenty-five mycobacterial strains isolated from tuberculosis patients in Burma at the National Tuberculosis Laboratory in Rangoon were subcultivated on Loevenstein-Jensen medium poured in universal glass containers and taken to Prague where after incubation at 37 °C for 14–21 days, new subcultures were made on L-J solid egg and in Šula's liquid medium. Some of the original cultures were lost due to the contamination which occurred in spite of the elimination of condensation water before transport of the containers. Thus, for final examination in thin colonial sections 16 subcultures 4 weeks old remained. They were further prepared for microscopic examination according to the technique previously described [3].

In short, the cultures are sterilized in 10% formal for 2 h, the suitable colonies are cut off and transferred in Petri dishes with 2% melted agar. To protect the surface of the colonies, another layer of melted agar is added and after solidification the agar blocks containing small piece of egg medium with the colony are processed in the same way as other histologic specimens. For cultures grown in the depth, the liquid medium was first pipetted off and the colonial deposit on the bottom of the tube was mixed with 5 ml of a melted 2% agar. By slowly rotating the tube in an oblique position the colonies were concentrated in the centre of the agar and after solidification the specimen was processed in the same way as stated above. The paraffin embedded colonies were cut by a Reichert microtome.

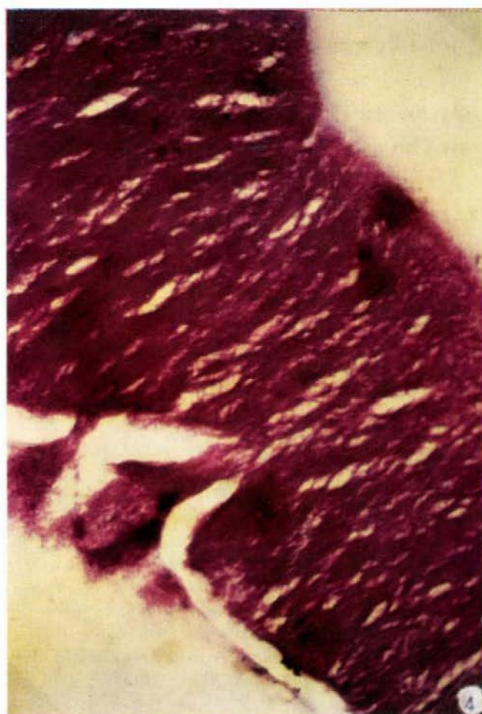
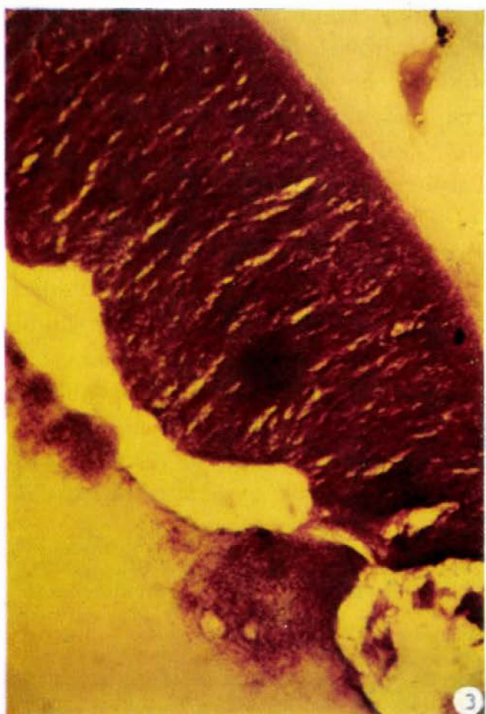
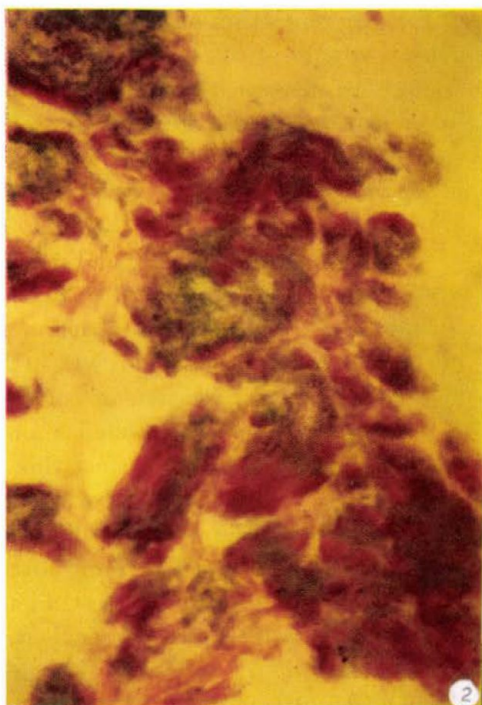
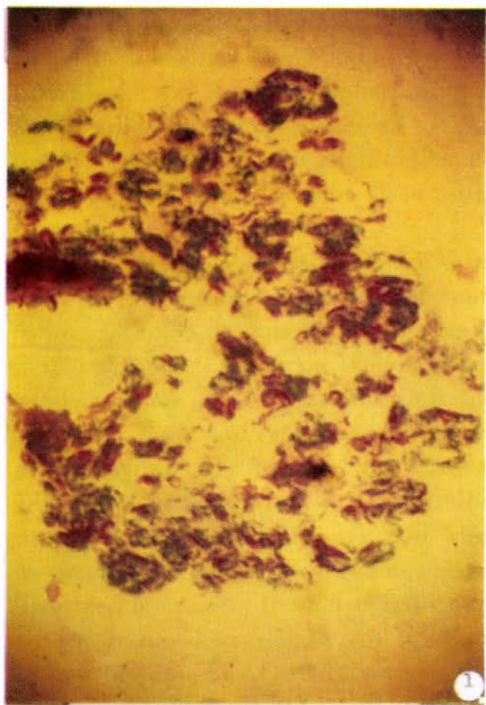
Altogether we studied several hundred sections taken from different parts of the same colonies so that the peripheral zones and central parts were cut and stained by the Ziehl-Neelsen technique.

Results and discussion

The results are demonstration in colour microphotographs (Plates I and II).

The technique of thin colonial sections is not widely used for examination of the microscopic texture of colonies. This is due partly to the complicated procedure requiring a skilled laboratory technician capable of making suitable colonial section of such a fragile material as are mycobacteria. On the other hand, gross examination of the surface of mycobacterial colonies is a well-established technique used by many authors for the taxonomy of mycobacteria

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Plate I. Figs 1 and 2. The growth pattern of Burmese strains in a deep culture of Šula's liquid medium composed of acid-fast cords loosely connected and non acid cyanophilic substance. $\times 60$ and $\times 900$. *Figs 3 and 4.* Vertical sections of surface colonies after 1 month growth on Loewenstein-Jensen medium. The colonies are composed of tightly packed acid-fast cords and clumps of mycobacteria penetrating into the subsurface layers of the egg medium. In a large clump on the right side two empty plaques are seen, indicating the possible presence of mycobacterial phages. $\times 600$ and $\times 800$

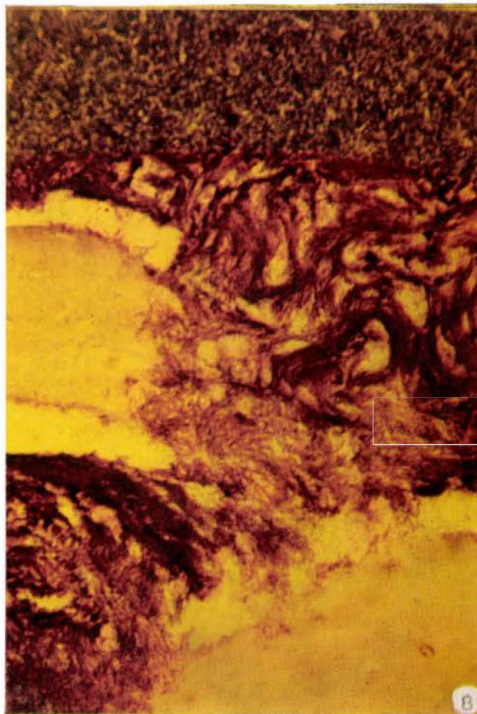
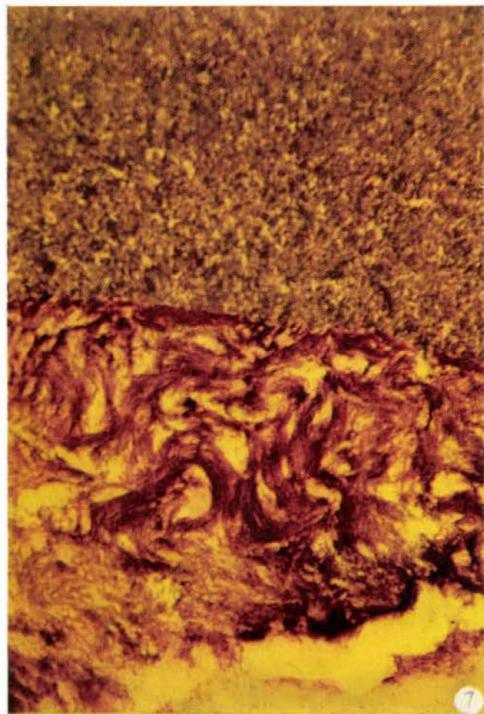
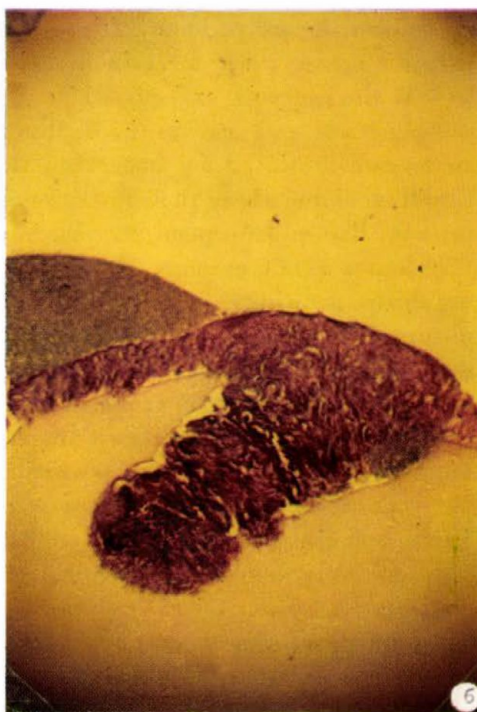


or for differentiation of suitable colonies for growing the most potent immunogenic variants of *M. bovis* BCG [4]. This technique, however, is not efficient enough to disclose the inner structure of the colonies which may be useful for a better differentiation of mycobacteria, especially for establishing intra-species variations where our techniques, with the exception of phage typing of *M. tuberculosis* and the serological analysis of *M. avium* and *M. intracellulare*, are still far from perfect.

In this sense systematic investigation of the microscopic texture of the mycobacterial colonies may be rewarding. Thus, we have been able by this technique to demonstrate hitherto unknown phenomena of colonial phage microlysis and subsurface growth in *M. bovis* and *M. africanum*, acid-fast, non acid-fast and stratification of colonies of the Connaught BCG strain [5]. In *Mycobacterium simiae* Weiszfeiler a clear demarcation was observed. This was formed by a thin non acid-fast line between the basis of the colony loosely connected with the surface of the medium with acid-fast "cogs" and the central parts of the colony. All these new features of mycobacterial growth could not be discovered without cutting the colonies and staining the sections.

The studies of Burmese strains are a further contribution to our knowledge of the micromorphological structure of mycobacteria isolated in the tropics. In general we could demonstrate among these 16 examined strains three different types of colony grown on L-J medium. The first type is practically identical with the European human strains composed of strong acid-fast densely packed cords strictly limited in their growth capacity to the surface of L-J medium. A subtype was found, having the same texture and staining properties but possessing in addition the capacity of subsurface growth located in the superficial layers of the egg medium. This growth pattern is typical for African strains. The third type is a mixed form composed of granular faintly acid-fast surface colonies the inner part of which is formed by strong acid-fast cords penetrating deeply into the egg medium. What is most surprising is the clear-cut borderline between those two parts of the same colony. A sudden change between the two kinds of mycobacteria forming the same colony would indicate a sudden change in their metabolism in particular of the lipids, because of the difference in acid-fastness. The possible explanation would be that the more remote parts of the colony are exposed to some nutritional deficit being located at a greater distance from the surface of the medium and having thus a diminished capacity of synthesizing energetically fastidious

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Plate II. Figs 5 and 6. Vertical section through the surface and subsurface colonies. The colonies consist of two different parts. The surface colony is composed of a granular faintly acid-fast mass with no definite mycobacterial texture. On the base of the colony, growth of distinct loosely connected cords occurs, penetrating into the deep layers of the egg medium. × 200 and × 400. Figs 7 and 8. Details of the colony seen in Fig. 5 at high power × 800. Clearly visible borders between the granular and cord-like colony. The cord-like colony grows deeply into the egg medium



lipids. On the other hand, this special kind of mycobacterium composed of mixed colonies must have in addition a special enzymatic system enabling to lyse the egg-yolk and egg-white of the medium, otherwise the germs could not penetrate and grow in the depth of the egg medium. This hypothesis seems to be substantiated by observing the growth of Burmese mycobacteria in liquid medium, where their nutritional requirements are fully satisfied and the growth, like of European strains, is quite uniform, composed of cords acid-fast and a small amount of cyanophilic substance, the matrix of acid-fast organisms growing in liquid media. Fully non acid-fast colonies, as previously observed in the *M. bovis* Connaught BCG strain witnessing some genetic deficit in certain bacterial cells, have not been found but their existence cannot be excluded. Due to this great variation of microcolonial texture, the Burmese mycobacteria, or at least those displaying subcolonial growth are very close to *M. bovis* or *M. africanum*, in which subsurface growth is a constant property.

Concerning the morphology of the rods, no "tadpole" bacilli were found in the colonies grown either on solid L-J or in liquid Šula's medium. Most of the rods were densely or loosely packed into the cords, the individual bacilli uniformly stained and their granules restricted to one or the other pole. The morphology of mycobacteria is variable and much depends on the environmental factors of growth, which are certainly different in artificial cultures as well as in the human body. This fact may explain the absence of "tadpole" bacilli in our experiments.

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RELIABILITY OF THE RABBIT PYROGEN TEST AND OF THE LIMULUS TEST IN PREDICTING THE PYROGENICITY OF VACCINES IN MAN

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Commercial vaccines including bacterial as well as live and inactivated viral vaccines were examined in the rabbit pyrogen test and in the limulus test. The laboratory results were compared with the reactivity in humans. A fairly good correlation was found between the temperature rise in rabbits and the frequency of febrile reaction in the vaccinees. Two rabies vaccines and a tick-borne encephalitis virus vaccine, each pyrogenic both in rabbit and in man, were negative in the limulus test. The pyrogenicity of these vaccines is attributed to the vaccine virus itself.

Parenteral pharmaceutical preparations have been examined for pyrogenicity in laboratory tests for several decades. The rabbit pyrogen test has been generally accepted for this purpose and the limulus amoebocyte lysate (LAL) test [1] is now used more and more frequently. The essence of the LAL reaction is that the amoebocytes of *Limulus polyphemus* contain a protein which is coagulated by minute amounts of endotoxin. The LAL test is strictly endotoxin-specific while substances of other chemical nature may also cause fever in rabbits. Since the pyrogenicity of injected preparations is usually due to an endotoxin contamination, a positive LAL reaction is a fairly good indicator of pyrogenicity.

Recently, a number of reports have been published on the pyrogen control of vaccines [2–9]. The rabbit test and/or the limulus test were used in these examinations. The vaccines prepared from Gram-negative bacteria, i.e. those with endotoxin as an inherent component, all proved positive in both tests. Furthermore, some purified polysaccharide and virus vaccines were found positive in both tests, indicating that these vaccines had been contaminated with endotoxin during preparation.

Data are scarce on the correlation between the laboratory results and the pyrogenicity of vaccines in man. KURONEN *et al.* [7], examining meningococcal polysaccharide vaccines found a good correlation between pyrogenicity in rabbits and LAL positivity on the one hand and the febrile response of the vaccinated children on the other. KREEFTENBERG *et al.* [8] found the LAL test suitable for predicting the pyrogenicity of typhoid vaccines in man. KNIGHT and LUCKEN [6] consider the rabbit pyrogen test reliable, but the LAL test unreliable, in testing bacterial vaccines. Finally, ENNIS *et al.* [4]

accept neither of the tests as a reliable indicator of clinical pyrogenicity of inactivated influenza vaccines.

The low number of observations and the contradictory results make it difficult to draw conclusions on the usefulness of pyrogen testing of vaccines. This study was undertaken to obtain further data about the reliability of the LAL test and that of the rabbit pyrogen test in prediction of the pyrogenic effect of vaccines in human.

Materials and methods

Vaccines. The Dipete, the typhoid and the influenza vaccines examined by us were Hungarian products while the measles, the tick-borne encephalitis (TBE), the mumps, the rabies and the rubella vaccines were purchased from abroad (Table II).

Pyrogen test. The test was performed as prescribed in the VIth Hungarian Pharmacopoea for the control of injection preparations [10]. Body temperature was taken by using a Universal Thermometer ELLAB apparatus. The rabbits included in the experiments were of 1500–2000 g body weight with a body temperature ranging between 38.5 and 39.5 °C on the day preceding the day of the experiment. The temperature measured in the morning of the day when the rabbits were injected with the vaccine, was accepted as the basal temperature. Each rabbit was injected with 1 human dose of vaccine per kg body weight. Three rabbits were injected with each vaccine. The adsorbed vaccines were centrifuged and the clear supernatants were injected. The temperature of the rabbits was measured 1, 2 and 3 h after vaccination. The vaccine was regarded as non-pyrogenic if none of the three rabbits developed a temperature rise exceeding 0.5 °C and the sum of the highest individual temperature rises ($\Sigma\Delta x$) did not exceed 1.2 °C. A preparation was qualified as pyrogenic if the temperature rise developed by one or more animals exceeded 0.5 °C and the $\Sigma\Delta x$ value exceeded 2.6 °C. If a preparation could not be qualified on the basis of the three-rabbit test, testing was continued using further 6 rabbits. The vaccine was qualified as non-pyrogenic if not more than three of the nine rabbits developed temperature rise exceeding 0.5 °C and the $\Sigma\Delta x$ value did not exceed 4.4 °C. Otherwise, the preparation was declared pyrogenic.

The LAL test. The "Pyrogent" kit (Mallinckrodt) was used. This consists of lyophilized limulus amoebocyte lysate, test tubes and lyophilized *E. coli* endotoxin. The lysate was reconstituted in pyrogen-free distilled water and 0.1 ml of the reconstituted lysate and the same volume of the vaccine under testing were measured into each test tube. The vaccines containing adsorbent were tested in their original form and the clear supernatant obtained by centrifugation was also examined.

Controls. (a) Negative water control: a mixture of 0.1 ml lysate and 0.1 ml distilled water; (b) positive water control: a mixture of 0.1 ml lysate and 0.1 ml endotoxin (50 ng/ml).

The mixtures were incubated in a water bath of 37 °C for 1 h. The test was considered valid if the negative water control had remained fluid while the positive water control had solidified. A vaccine was regarded as LAL-positive if, like the positive water control, the vaccine + lysate mixture coagulated. From each LAL-positive preparation, a tenfold dilution series was prepared and tested. The highest dilution that coagulated the lysate was regarded as the LAL titre.

If an undiluted vaccine was found LAL-negative, it was subjected to a positive sample control to show any substance inhibiting the LAL reaction. Positive sample control = 0.1 ml endotoxin (50 ng/ml) diluted in the vaccine to be tested + 0.1 ml of the lysate.

The preparation was accepted as LAL-negative if it did not inhibit the coagulation of the positive sample control.

Potency tests. Test for pertussis vaccine: the intracerebral mouse-protective test was applied using a national reference preparation calibrated to the International Standard Pertussis vaccine [11]. Test for rabies vaccine: the NIH test [12] was applied using a national reference sheep brain vaccine calibrated to the International Standard Rabies Vaccine.

Registration of vaccinations and vaccine reactions. The febrile reactions appearing within 48 h after vaccination and lasting 1–2 days were registered after the following vaccinations: the first injection of the Dipete basal immunization of 3-month-old infants; influenza vaccina-

tion of adults; the first injection of a postexposure series of rabies vaccination of adults; the first injection of vaccination against TBE of adults.

The reactions were registered by the physician or the nurse as stated by the vaccinee or his caretaker.

Results

1. Sensitivity of the pyrogen test and of the LAL test, using *Escherichia coli* endotoxin.

A tenfold dilution series of the endotoxin included in the Pyrogen kit was subjected to both tests (Table I). Pyrogen-free distilled water was used as diluent. The lowest endotoxin concentration detectable by the rabbit test and the LAL test was 5–50 ng/ml and 0.05–0.5 ng/ml, respectively, i.e. the LAL test was about 100 times as sensitive as the pyrogen test.

Table I

Sensitivity of the rabbit pyrogen test and of the limulus test in the detection of E. coli endotoxin

<i>E. coli</i> endotoxin ng/ml	Results of the	
	rabbit pyrogen test*	limulus test
500	pyrogenic	
50	pyrogenic	
5	non-pyrogenic	+
0.5	non-pyrogenic	+
0.05		—
0.005		—

* Dose: 1 ml endotoxin solution/kg body weight

2. Results of the LAL test, pyrogenicity in rabbit and pyrogenicity in man

The results are reviewed in Table II. In the column "Pyrogenicity", besides presenting the pharmacopoeal qualifications, the average of the maximum temperature rises of the vaccinated rabbits ($\overline{\Delta x}$) are given as additional data.

2.1. *Correlation between the LAL test and the pyrogen test.* All the preparations could be examined in the LAL test, i.e. none of them contained any substance inhibiting coagulation of the lysate. Since the rabbits did not tolerate the intravenous injection of adsorbed vaccines, these were centrifuged and the adsorbent-free supernatants were injected. The LAL titre of the supernatant was 1 or 2 log units lower than that of the original vaccine.

Table II

LAL titres and pyrogenicity for rabbit and man of commercial vaccines

Vaccine	Manufacturer	Series No.	LAL titre	Pyrogenicity			
				in rabbit		in man	
				pharmacopoeal qualification	$\overline{\Delta x}$	No. of vaccinees observed	No. of febrile responses ($\geq 38^\circ\text{C}$)
<i>Bacterial vaccines</i>							
Dipete AlPO ₄ -adsorbed	HUMAN, Budapest	119 □	10^{-5} 10^{-3}	pyrogenic	1.1	106	3
		126 □	10^{-4} 10^{-3}	pyrogenic	1.9	179	23
		128 □	n. t. 10^{-4}	pyrogenic	1.7	80	5
Typhoid—tetanus	HUMAN, Budapest	L. 116	10^{-5}	pyrogenic	2.2	n. o.	
<i>Live virus vaccines</i>							
Mumps	Behringwerke, Darmstadt	14002	<1	non-pyrogenic	0.03	338	—
		14003	<1	non-pyrogenic	0.1	4597	7
Measles	Institute of Viral Preparations Moscow	628	10^{-1}	non-pyrogenic	0.2	n. o.	
Rubella	RIT, Genval Wellcome, UK	4E21Re23/75F06	<1	non-pyrogenic	0.03	n. o.	
		Ru/10/20	<1	non-pyrogenic	0.2	n. o.	
<i>Inactivated viral vaccines</i>							
Influenza A AlPO ₄ -adsorbed	National Institute of Hygiene, Budapest	63	<1			64	—
		□	<1	non-pyrogenic	0.03		

Influenza B AlPO ₄ -adsorbed	National Institute of Hygiene, Budapest	64 □	10 ⁻¹ 10 ⁻¹	non-pyrogenic	0.0	n. o.	
Rabies HDC	Mérieux, Lyon	N 0335 - B × × R0487 × ×	<1 <1	pyrogenic pyrogenic pyrogenic non-pyrogenic	1.9 1.6 0.7 0.3	15 n. o.	6
Tick-borne encephalitis virus vaccine AlOH ₃ - adsorbed	Immuno, Austria	370778 □ 370379 □	10 ⁻¹ <1 10 ⁻¹ 10 ⁻¹	pyrogenic pyrogenic non-pyrogenic	0.5 0.1	66 589	1 —

n. o. = not observed

n. t. = not tested

□ = clear supernatant after centrifugation

× × = 0.2 ml/kg body weight

Δx = average maximum individual temperature rise

The bacterial vaccines had high LAL titres, *viz.* 10^{-3} – 10^{-5} . The supernatants of the Dipete vaccines also exceeded the 10^{-3} value. All these preparations proved pyrogenic in the rabbit, in accordance with the 100-fold sensitivity of the LAL test.

The live virus vaccines were either LAL-negative or reacted only undiluted. None of these preparations were pyrogenic, indicating a good accordance of the LAL and the rabbit tests in this group of preparations.

In the group of inactivated virus vaccines, there was a good correlation between the LAL test and the rabbit pyrogen test in case of the influenza vaccines and of the TBE vaccine No. 370379. Unlike these, both the rabies vaccines and the TBE vaccine No. 370778 proved negative in the LAL test and pyrogenic in the rabbit test. Rabies vaccine No. N 0335–B appeared more pyrogenic than did No. R 0487. The human dose (0.5 ml) of the former induced in rabbits an average temperature rise of 1.9°C and the preparation was pyrogenic even in dose of 0.2 ml/kg. The human dose of the latter vaccine induced an average temperature rise of 0.7°C , and this preparation was non-pyrogenic in the dose 0.2 ml/kg.

2.2. *Relationship between the results of the laboratory tests and the febrile reaction of the vaccinees.* The pyrogenic effect in humans was registered for 9 of the 15 preparations examined in the laboratory. There was a good accordance between the human reactivity and the results of the rabbit pyrogen test. The preparations that had caused fever in a smaller or larger proportion of the vaccinees, except mumps vaccine No. 14003, were pyrogenic for rabbits as well. Vaccine No. 14003, non-pyrogenic in rabbits, caused fever in as few as 7 of the 4597 vaccinees (0.2%). The vaccines nonreactive in human vaccinees

Table III
Pyrogenicity of vaccine preparations in rabbit and man

Vaccine	Series No.	Temperature rise in rabbits	Febrile response in vaccinees	
		Δx	reaction/vaccinated	per cent
Mumps	14002	0.03	0/338	0
Influenza A	63	0.03	0/64	0
Tick-borne encephalitis	370379	0.1	1/589	0.2
Mumps	14003	0.1	7/4597	0.2
Tick-borne encephalitis	370778	0.5	1/66	1.5
Dipete	119	1.1	3/106	2.8
	128	1.7	5/80	6.3
	126	1.9	23/179	12.8
Rabies HDC	N 0335 – B	1.9	6/15	40

Δx = average maximum individual temperature rise

were all non-pyrogenic in the rabbit test. The correlation between the LAL test and the human pyrogenicity was, of course, satisfactory only in those cases in which there was a good correlation between the two laboratory tests.

Table III includes only those vaccines for which the human reactivity was determined. The order of the preparations arranged according to their Δx values was the same as their order according to the frequency of the febrile response by vaccinees. (Kendall's rank correlation coefficient: 0.92).

3. Potency of the pertussis component of Dipete vaccines

The potency of the pertussis component in 1 ml of the vaccines Nos 119, 126 and 128 proved to be equivalent to 6.7, 8.4 and 7.8 IU, respectively.

4. Potency of rabies vaccines

The antigenic values were as follows. Vaccine No. N 0335—B: 12.5; vaccine No. RO487: 1.8.

Discussion

Our observations suggest that the pyrogen test in rabbits is reliable for predicting the human pyrogenicity of vaccines. The order of the vaccines is the same if arranged according to the average maximum temperature rise (Δx) induced in rabbits or according to the frequency of a febrile response in human vaccinees (Table III). One may therefore suggest that man and the rabbit are sensitive to the same pyrogen(s) present in human vaccines. Quantitative conclusions cannot be drawn because the vaccines examined by us were highly variable in type, and the groups of vaccinees were different in both size and age. Thus, the expectable frequency of febrile reaction in human vaccinees cannot be calculated simply from the temperature rise measured in rabbits.

On the other hand, we suppose that the Δx value may provide useful information when the least reactive is to be chosen from different series of a given vaccine. Table III shows that the three Dipete series were different in pyrogenicity and that the differences manifested themselves equally in rabbits and infants.

BROWER and COHEN [13] examined pertussis vaccines prepared by acid precipitation and centrifugation. They found differences between the vaccines in human reactivity. Although the Dipete vaccines examined by us had been prepared by a consistent technology, the variable reactivity of the vaccines is not surprising if the biological complexity and variability of *Bordetella pertussis* is considered. The existence of differences between the three series is

supported by their different immunogenicity values in the active mouse-protective test, equalling 6.7 (No. 119), 7.8 (No. 128) and 8.4 (No. 126) IU/ml.

The LAL test as a simple and sensitive test for the detection of endotoxin has been recommended to replace the rabbit pyrogen test. The field of application of the LAL test is, however, limited by its strict endotoxin-specificity; non-endotoxin pyrogens cannot be detected by this test. Based on experience with rabies and TBE vaccines we assume that the LAL test should be used not instead of, but parallel with, the rabbit test in the control of vaccines except those prepared from Gram-negative bacteria. Co-evaluation of both tests may provide information not obtainable from either of the tests. According to the data presented in Table II, both rabies vaccines proved pyrogenic, though neither was LAL-positive. The seemingly contradictory results indicate that the pyrogenicity of the preparations was due to some non-endotoxin pyrogen, presumably to the vaccine virus itself. This view is supported by the fact that vaccine No. N 0335—B, which in the rabbit was more pyrogenic than vaccine No. R0487, immunized experimental animals to a higher degree against a challenge with the CVS virus than did the latter vaccine. In the NIH-type mouse-protective test [12], the antigenic value of the N0335 series was found 12.5 while that of No. R 0487 1.8. It seems therefore reasonable to assume that the striking pyrogenic effect and the exceptionally strong immunogenicity of the former series were due to the same factor, *viz.* the unusually high virus content of the series. As to human reactivity, we succeeded in collecting data for series N0335—B, but not for R 0487. As Table II shows, series N0335—B was strikingly reactive. Experience similar to our own has been reported by KUWERT *et al.* [14], but these authors attributed the pyrogenicity to endotoxin contamination. In our study, the negative LAL test indicated the non-endotoxin origin of the pyrogenicity.

Like the rabies vaccine the TBE vaccine No. 370778 was found pyrogenic in the rabbit test and negative in the LAL test. This preparation induced a febrile response in only 1 of 66 vaccines, but the single response was a typical vaccination reaction, *viz.* the temperature rise accompanied by severe prostration, reached 39.1 within 24 h and lasted less than 24 h. Presumably, the reaction was caused by the vaccine virus itself in this case, too.

Finally, our observations call attention to the importance of viral pyrogens in the reactivity of inactivated virus vaccines. The pyrogenicity of influenza virus was widely examined in the sixties [15] but the last decade has brought little progress in this field. In our small material we found three inactivated virus vaccines the pyrogenicity of which were attributed not to endotoxin contamination, but presumably to the incorporated virus strain.

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INULINASE ACTIVITY OF *PICHLA POLYMORPHA*

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Pichia polymorpha has two inducible inulinases of the β fructosidase type. The enzymes are synthesized in the cell wall, but are easily excreted into the culture medium. The enzymes differ only in their optimum pH, and one of the two contains some carbohydrate residues.

Inulin is the sugar reserve of some compositae: Jerusalem artichoke (*Helianthus tuberosus*), dandelion (*Taraxacum dens leonis* Desf.), chicory (*Cichorium intybus* L.), etc. The fructose polymer can be hydrolyzed by inulinases. Many of them belong to the group of β fructosidases. In the Jerusalem artichoke the inulinase activity is distinct from invertase activity: the inulinase has no action upon sucrose [1]. The other inulinases, of the β fructosidase type, are active on both substrates. They have been described in various organisms: moulds [2, 3] and yeasts. *Kluyveromyces fragilis* [4, 5], *Candida salmanticensis* [6], *Candida kefir* [7], *Debaryomyces cantarellii* [8] have an inducible inulinase. Some authors mention the presence of an extracellular enzyme and an intracellular one, with similar biochemical properties: *Candida kefir* [7]; *Kluyveromyces fragilis* [4]. These enzymes catalyze the hydrolysis of inulin by splitting off terminal fructosyl units (D-fructose).

The process is different for *Aspergillus niger* [9] and *Arthrobacter ureafaciens* [10] which hydrolyze inulin, liberating oligosaccharides and difructose anhydride, respectively.

Materials and methods

Microorganism. *Pichia polymorpha* CBS 186, from the Centraalbureau voor Schimmelcultures, Delft, Holland, was used.

Cultural conditions. Unless stated otherwise, the medium (Yeast Nitrogen Base, Difco) contained 0.5% inulin (Merck). Erlenmeyer flasks were filled to $\frac{1}{10}$ volume and shaken (1.3 hertz, amplitude 75 mm). The populations were evaluated by haematimetric counts, or by nephelometry at 400 nm, or by a protein test according to STRICKLAND [11].

Cell-free preparations. Crushed cells were prepared in a Braun MKS apparatus [12]. Protoplasts were prepared according to DARLING *et al.* [13]. The cell-free extracts were fractionated by centrifugation (Fig. 1).

Enzyme activity test. The activity on substrates was determined directly by reducing sugar test according to SOMOGYI [14]. Unless stated otherwise the reaction mixture (volumes,

2 ml), contained 0.25% inulin, 0.01 M buffer and the enzyme. The reaction was stopped by adding 2 ml of Somogyi's reagent. Specific estimation of glucose and fructose was done using the enzymatic method of BERGMEYER *et al.* [15]. The reducing fructosides were detected by

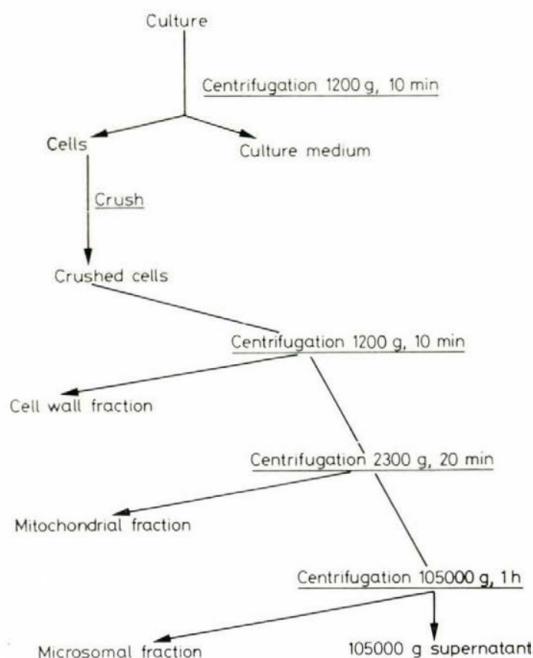


Fig. 1. Scheme of fractionation method for cell-free preparations

paper chromatography [4]. Enzyme units are expressed as μ moles of fructose liberated per minute at 25 °C. Specific activities are expressed in units/ml of protein, tested by the method of LOWRY *et al.* [16].

Results

1. Characterization and localization of the activity

After growth on YNB inulin medium for 48 h, the inulinase activity was sought in various fractions. More activity was present in the culture medium than in the undamaged cells. Crushing the cells increases the activity considerably. After fractionation of this extract, the activity was recovered in both the sediment at 1200 g (cell wall fraction) and the supernatant of the 105 000 g centrifugation (Table I). The protoplasts and the burst protoplasts had no activity. These results tend to show that the enzyme was partly cryptic in the cell wall, and released readily into the culture medium.

The hydrolytic action of the various preparations on sucrose was also studied, with similar results. The I/S ratios (inulinase activity/invertase activ-

Table I
Localization of the enzyme

Fractions	Activity in per cent of cell activity	Specific activity (units/mg protein)	I/S ratio
Cells	100	0.01	0.065
Crushed cells	360	0.033	0.16
Cell wall fraction	94	0.011	0.061
Mitochondrial fraction	0	0	—
Microsomal fraction	0	0	—
105 000 g supernatant	170	0.03	0.17
Culture medium	170	0.039	0.068
Protoplasts	0	0	—
Burst protoplasts	0	0	—

ity) were calculated for the various preparations (Table I). Crushing led to a greater activity for inulin (360% of the activity before crushing) than for sucrose (125%). A steric impediment as regards inulin may occur for the cell wall enzyme.

BELUCHE *et al.* [8] have demonstrated with *Debaryomyces cantarellii* that it is possible to extract the cell wall enzyme with a buffer (0.02 M pH 8.5 phosphate). In these conditions the extracted activity for *Pichia polymorpha* is superior to the activity of intact cells. When the cells grow in buffered medium, the extractable activity is the same as with an unbuffered one, whatever the pH (3.5; 5.5; 7).

2. Purification of the enzymes

The culture medium was dialyzed. It had a similar activity on inulin and sucrose as a function of pH (Fig. 2). Two distinct maxima are shown, one sharp (2–2.5 pH) the other broad (4–5.5), suggesting the presence of two enzymes.

The inulinase activity was all retained on a DEAE cellulose column, with 0.1 M pH 5 acetate buffer. The column was eluted with a linear gradient of NaCl (0 to 1 M). The whole activity was eluted at 0.3 NaCl. Continuous elution with 0.3 NaCl led to separation of the activity into two fractions (Fig. 3). After concentration with an Amicon cell (with a PM 10 Diaflo membrane), the culture medium was eluted from a Sephadex G 150 column with 0.1 M pH 5 acetate buffer. Separation into two fractions appeared (Fig. 4). The activity curves in relation to the pH on inulin similarity between the first fractions obtained on DEAE cellulose and Sephadex. This is also true for the two second

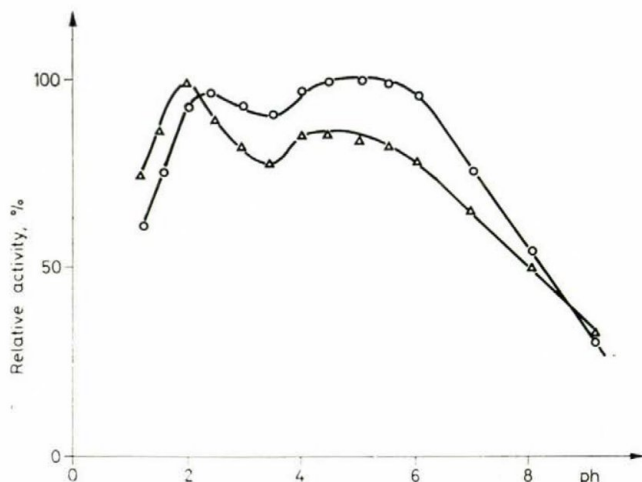


Fig. 2. Effect of pH on the activity of the culture medium; Δ inulin, $\circ$ sucrose

fractions. Moreover, the first fraction of DEAE cellulose was eluted on Sephadex in a single fraction which appeared on the graph as the first fraction eluted from the culture medium. The second fraction from DEAE cellulose when eluted on Sephadex appears in the same place as the second fraction eluted from the culture medium.

Thus *Pichia polymorpha* has two inulinases; they will be referred to as enzyme EI and enzyme EII in order of their elution.

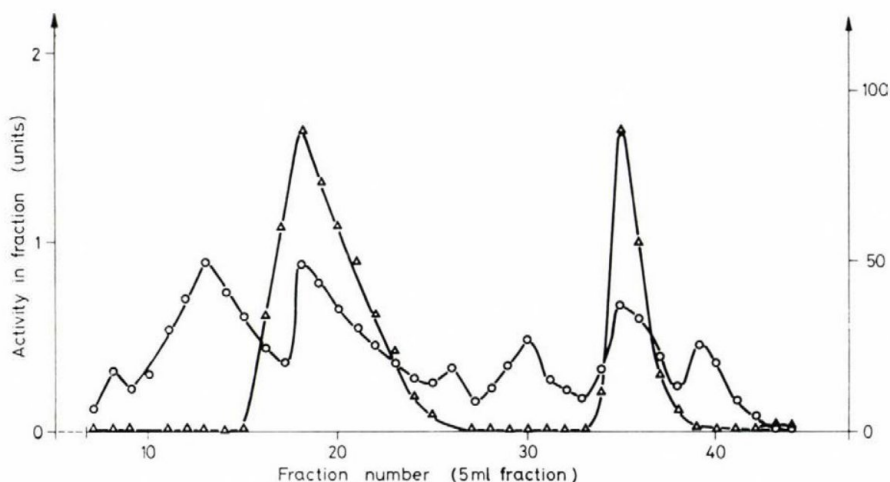


Fig. 3. Chromatography of the culture media on DEAE cellulose with a 0.1 M pH 5 acetate buffer, and 0.3 M NaCl; Δ inulinase activity, $\circ$ protein concentration

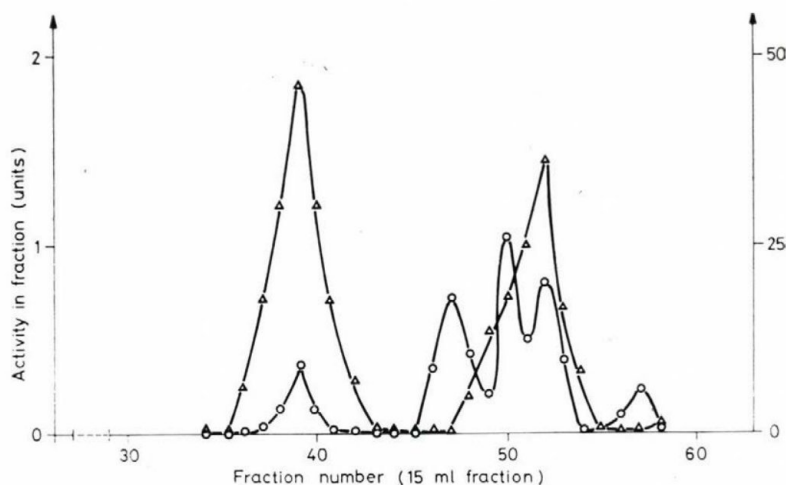


Fig. 4. Chromatography of the culture medium on G 150 Sephadex with an 0.1 M pH 5 acetate buffer; Δ inulinase activity, $\circ$ protein concentration

3. Biochemical properties of the two enzymes

Biochemical properties of the enzymes were studied on three substrates: inulin, sucrose and raffinose.

Study of enzyme EI. Effect of pH. The curves on the three substrates are similar (Fig. 5). The optimum pH is 2–2.5.

Effect of temperature. Figure 6 shows the influence of temperature on the activity. The optimum temperature is 45 °C for the three substrates. The

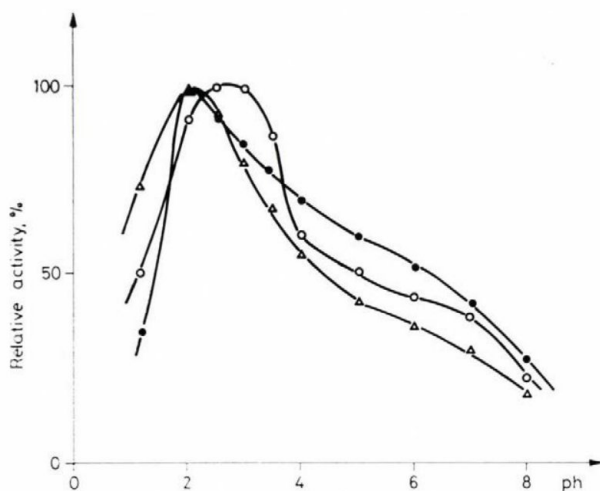


Fig. 5. Effect of pH on enzyme EI activity with various substrates; Δ inulin, $\circ$ sucrose, $\bullet$ raffinose

maximum velocity (V_m) was determined to allow a logical comparison between the three substrates: they are obtained from the initial velocities measured under standard conditions by extrapolation of Lineweaver and Burk's curves. The specific activities evaluated with this method were 0.2 mM fructose/min/mg protein for inulin, 3.2 mM/min/mg for sucrose, and 0.6 mM/min/mg for raffinose. Figure 7 shows the effect of higher temperatures upon enzyme stabil-

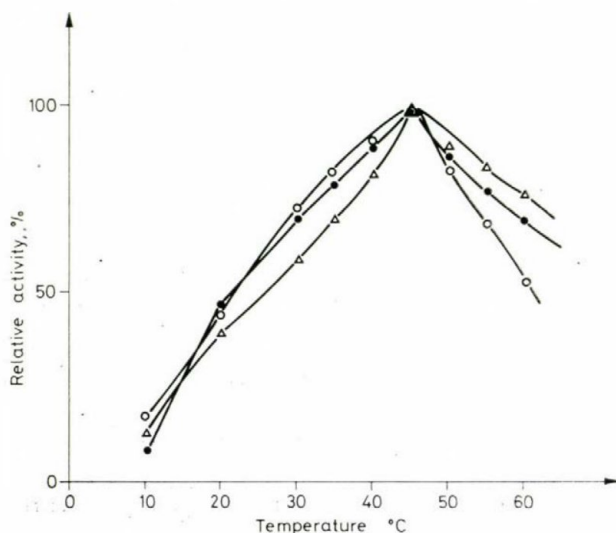


Fig. 6. Effect of temperature on enzyme EI activity, at initial velocity (5 min), with various substrates; Δ inulin, $\circ$ sucrose, $\bullet$ raffinose

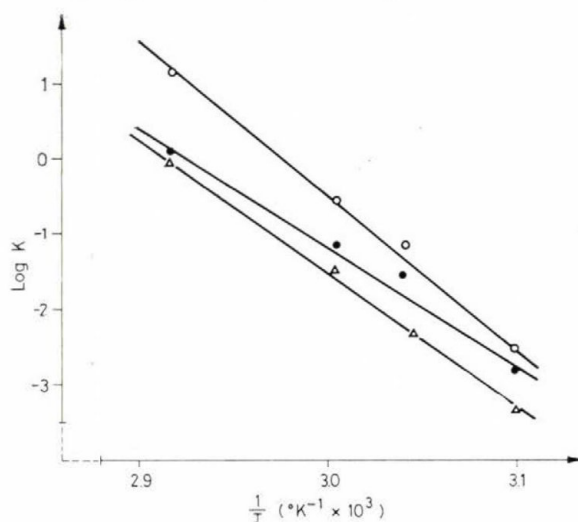


Fig. 7. Inactivation of enzyme EI activity by high temperatures measured with various substrates; Δ inulin, $\circ$ sucrose, $\bullet$ raffinose, (k : thermal denaturation rate)

ity. The enzyme was denatured at 70 °C in 5 min. The activation energies of thermic denaturation for EI were: 35 kcal/M for inulin, 44 kcal/M for sucrose, and 36 kcal/M for raffinose.

Influence of effectors. Table II summarizes the results obtained. Metals of high molecular weight and NBS are good inhibitors. Inulinase activity seems to be more sensitive than the others.

Table II

Influence of effectors on enzyme EI activity with various substrates

Effectors	Per cent of inhibition with					
	inulin		sucrose		raffinose	
	10 ⁻³ M	10 ⁻⁵ M	10 ⁻³ M	10 ⁻⁵ M	10 ⁻³ M	10 ⁻⁵ M
Ag (AgNO ₃)	100	74	100	55	21	0
Hg (HgCl ₂)	100	40	100	75	76	51
Mn (MnCl ₂)	94	0	10	0	90	55
Pb (PbNO ₃)	78	7	100	11	25	0
Cu (CuSO ₄)	100	42	45	0	15	0
Mg (MgCl ₂)	51	0	91	5	13	0
Fe (FeCl ₂)	87	0	56	0	36	0
Zn (ZnCl ₂)	0	0	0	0	11	0
Anhydride acetic	68	0	10	0	23	0
Malonitril	100	32	80	0	16	5
Pyridoxal	0	0	0	0	13	0
NBS	100	86	51	0	86	32

Mode of enzyme action. Free fructose appears during the enzymatic reaction, and free glucose appears at the end. No reducing polyfructosides appear. EI is thus a β fructosidase that hydrolyzes inulin into fructose and a non-reducing polymer until only glucose remains.

Specificity of the enzyme action. Michaelis' constants (Km) were) determined using Lineweaver and Burk's method: $2.5 \cdot 10^{-3}$ M for inulin, $2.9 \cdot 10^{-3}$ M for sucrose, and $3.6 \cdot 10^{-3}$ M for raffinose.

Observations regarding the structure of EI. After a very long non-stop dialysis of EI, the anthrone test [17] detected the presence of carbohydrate residues. After hydrolysis by N HCl for 30 min, thin-layer chromatography [18] showed the presence of glucose and mannose. The sugar protein ratio is 0.016%.

Study of enzyme EII

Effect of pH. The pH had the same effect on EII activity for the three substrates. The optimum pH is 4–5.5 for EII (Fig. 8).

Effect of temperature. The optimum temperature was again 45 °C for the three substrates (Fig. 9). The specific activities were, 0.05 mM of fructose/min/mg protein for inulin, 0.65 mM/min/mg for sucrose, 1.6 mM/min/mg for raffinose.

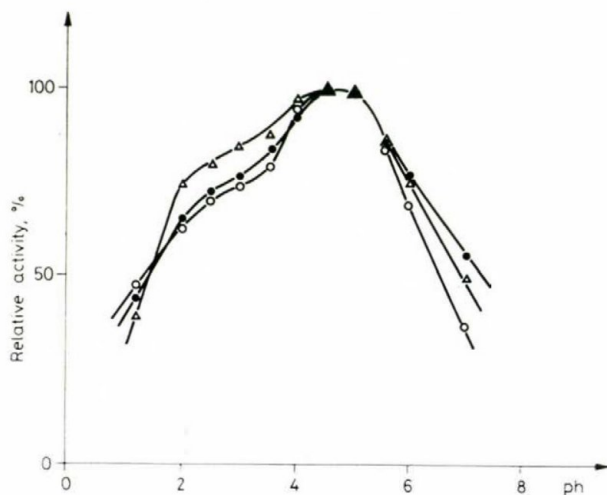


Fig. 8. Effect of pH on enzyme EII activity with various substrates; Δ inulin, $\circ$ sucrose, $\bullet$ raffinose

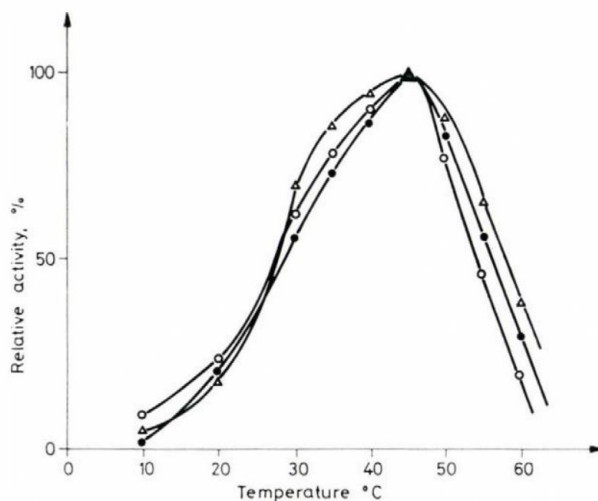


Fig. 9. Effect of temperature on enzyme EII activity at initial velocity (5 min), with various substrates; Δ inulin, $\circ$ sucrose, $\bullet$ raffinose

The same influence of high temperature was observed (Fig. 10). The activation energies of thermic denaturation (E_a) were: 11 kcal/M for inulin, 23 kcal/M for sucrose, and 24 kcal/F for raffinose.

Influence of effectors. Similar results as for EI were obtained (Table III).

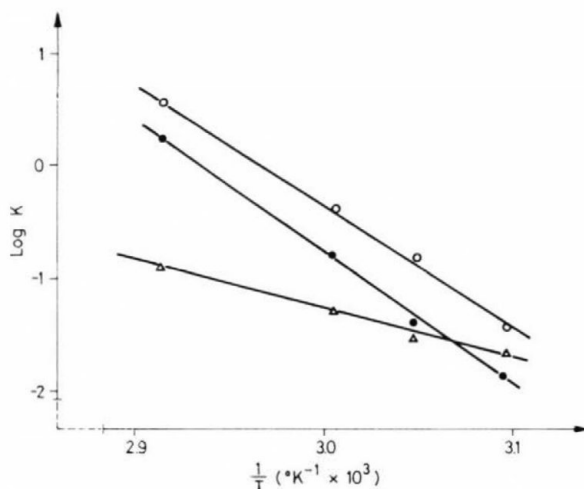


Fig. 10. Inactivation of enzyme EII activity by high temperatures measured with various substrates; Δ inulin, $\circ$ sucrose, $\bullet$ raffinose, (k : thermal denaturation rate)

Table III

Influence of effectors on enzyme EII activity with various substrates

Effectors	Per cent of inhibition with					
	inulin		sucrose		raffinose	
	10^{-3} M	10^{-5} M	10^{-3} M	10^{-5} M	10^{-3} M	10^{-5} M
Ag (AgNO_3)	100	75	60	30	75	38
Hg (HgCl_2)	100	46	100	50	82	47
Mn (MnCl_2)	80	27	79	47	86	26
Pb (PbNO_3)	64	31	100	48	100	5
Cu (CuSO_4)	88	3	20	0	33	0
Mg (MgCl_2)	90	21	20	0	36	0
Fe (FeCl_2)	100	7	20	0	43	23
Zn (ZnCl_2)	38	0	53	0	36	0
Anhydride acetic	68	0	10	0	23	0
Malonitril	100	18	100	13	100	86
Pyridoxal	0	0	0	0	0	0
NBS	100	28	100	53	100	36

Specificity of the enzyme action. The K_m was $1.3 \cdot 10^{-3}$ for inulin, $2.8 \cdot 10^{-3}$ M for sucrose, and $4.5 \cdot 10^{-3}$ M for raffinose.

Mode of enzyme action. The mode of enzyme action of EII upon inulin was the same as for EI.

Observation regarding the structure of EII. EII did not seem to have carbohydrate residues.

4. Effect of substrates on enzyme production

The inulinase activity was nil after a YNB 0.5% glucose culture. A pre-culture on this medium was used to sow some media containing various sugars (Table IV). Inulin induced biosynthesis in the cell wall, and excretion into the culture medium of both enzymes. Sucrose and raffinose had a weaker inducing effect: there was a slight biosynthesis of both enzymes in the cell wall, but excretion was nil. A glucose effect was noticeable; glucose and fructose partly inhibited the biosynthesis of both enzymes in the presence of inulin. Cells cultivated on glucose medium were transplanted onto media containing the

Table IV
Induction of synthesis of the two enzymes by various sugars

YNB + various sugars	Units per 100 ml of culture							
	Crushed cells				Culture medium			
	24 h		48 h		24 h		48 h	
	pH 2	pH 5	pH 2	pH 5	pH 2	pH 5	pH 2	pH 5
0.5% glucose	0	0	0	0	0	0	0	0
0.5% sucrose	4.0	1.7	0.9	0.7	0	0	0	0
0.5% raffinose	1.3	1.8	1.3	0.8	0	0	0	0
0.5% inulin	25.0	11.6	15.0	7.0	3.3	1.5	6.9	3.2
0.5% inulin + 0.5% glucose	1.3	1.6	4.6	3.9	0	0	0	0
0.5% inulin + 0.5% fructose	1.3	1.5	0.9	1.0	0	0	0	0

sugars mentioned above, but without YNB: i.e. without growth. In all cases, except with glucose, the enzymes appeared slightly in the cell wall but were not excreted.

Conclusion

Pichia polymorpha has an enzymatic complex containing two inulinases (type β fructosidase) hydrolyzing inulin, and freeing D-fructose. These enzymes are also able to hydrolyze sucrose and raffinose. The complex is situated in the

cell wall, but its excretion into the culture medium is easy. Synthesis of both enzymes can be induced by inulin, which facilitates excretion. Glucose and fructose partly inhibit the enzyme synthesis.

EI and EII have similar biochemical properties, and only seem to differ in their optimum pH, and the presence of glucidic residue for EI.

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PHARMACOKINETIC AND PHARMACOLOGICAL ASPECTS OF INTERFERON THERAPY IN MAN*

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Interferons have been given to experimental animals in order to determine their protective potential against virus infections and cancer. Similar experiments are now increasingly being performed on human patients. In this context, it would be valuable if well documented information on the tissue distribution, decay rate and metabolism of the injected material would be available. For several reasons, however, researchers have been withheld until now to explore this area extensively. The main reason for this is that interferons are only available in small quantities, so that, even after injection of the highest possible dose, barely detectable concentrations of interferon are present in the tissues. Furthermore, pure interferons are even more scarce and radioactively labelled interferons are until now unavailable in quantities suitable for pharmacokinetic evaluation.

The toxicology of interferon is a field as unexplored as that of its pharmacokinetics. Although interferon was initially advocated as a natural non-toxic antiviral substance, many side effects have been reported in treated patients. Whether these side effects are due to the interferon molecule itself or to certain impurities in the clinical preparations is still a matter of conjecture. The possibility must even be considered that not only the side effects but the so-called beneficial effects (e.g. antitumour effect) are due to impurities rather than to interferon itself.

Pharmacokinetics of human interferon as apparent from clinical studies

Leucocyte and fibroblast interferon preparations have been given by intramuscular injection to patients in order to evaluate their clinical effectiveness. During these experiments blood titres of interferon were determined [1]. In a systematic study GREENBERG *et al.* [1] reported maximum blood titres of ~ 100 units/ml in patients receiving a single dose of 3×10^6 units. In patients receiving daily injections the maximum titre was ~ 300 units/ml. Some

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investigators have also been able to recover interferon from cutaneous lesions [2], indicating that it could penetrate and persist for some time in tissues. Other studies [3] have not confirmed this finding. More detailed data on tissue distribution of leukocyte interferon in man are not available.

Fibroblast interferon has also been given by intramuscular injection to numerous patients by several research groups [4-13]. Low or undetectable levels of serum interferon were found despite the fact that the injected preparations were of similar potency as those of leukocyte interferon. A minimum of 10 to 30×10^6 units had to be given for interferon to become detectable in the serum [13].

Although there is evidence that spontaneous inactivation of interferon in body fluids occurs more rapidly with fibroblast than with leukocyte interferon [14, 15], there is no proof that this mechanism is sufficient to account for the observed differences in pharmacokinetics. Moreover, rapid inactivation of fibroblast interferon by body fluids has not been found in our laboratory (unpublished data).

Pharmacokinetics of human interferon in animal experiments

Various animal species have been used to study the pharmacokinetics of human interferon [4, 16-21]. Blood titres of leukocyte and fibroblast interferons were compared in rabbits injected intramuscularly. Early experiments [17] failed to reveal any difference in pharmacokinetic behaviour between the two interferons. The fibroblast interferon used for these experiments was obtained by superinduction in diploid skin muscle cells and had partially been purified by fractional ammonium sulphate precipitation, a method that yielded recoveries of around 10%. Later experiments [4] were performed with interferon that was also prepared from diploid cells but had been purified partially by adsorption-elution from controlled pore glass (CPG) beads, a method that routinely yields recoveries of over 50%. With this interferon a clear-cut difference with leukocyte interferon was observed in serum titres obtained after intramuscular injection to rabbits. Unexpectedly, the same preparations injected intramuscularly to monkeys, failed to reveal a significant difference: high serum titres were obtained with either leukocyte or fibroblast interferon [20, and unpublished observations of the author]. Hence, the rabbit was considered to be a better model for interferon pharmacokinetics in man. In an independent study, VILCEK *et al.* [19] confirmed that in rabbits intramuscular injection of fibroblast interferon resulted in lower blood titres than those obtained with a similar injection of leukocyte interferon. In this study the difference between the two interferons was, however, less pronounced. The fibroblast interferon used by these authors was also of diploid cell origin and had not undergone any purification.

Intravenous injections of leukocyte and fibroblast interferons in rabbits failed to reveal significant differences in clearance rate [4, 19]. It is doubtful, however, whether this experimental design was accurate and sensitive enough to reveal small differences that were sufficiently important to account for lower blood values after intramuscular injection. Thus, these experiments while failing to support the idea of a more rapid clearance of fibroblast type interferon, did not refute this possibility either.

The toxicology of human interferon

Interferon exerts a variety of effects on cells which suggest that treatment of intact animals or man with interferon may be accompanied by various side effects. From studies in mice it would appear that therapeutically effective regimens of interferon treatment are not toxic. Only when large doses of interferon were given to very young animals could any right-out toxicity be observed [22-24]. Although such high doses have not been used in man, distinct undesirable side effects of interferon therapy have been noted in man.

Fever. Most authors who have used leukocyte interferon to treat patients, mention mild to severe fever reactions [2, 3, 25]. These are most prominent after the first injection and tend to become less severe after prolonged treatment. The fever is usually accompanied by chills and malaise. Similar reactions were seen in some but not all patients treated with fibroblast interferon [4-7]. From a group of 27 patients [4, 26] who received intramuscular injections of fibroblast interferon (diploid cell interferon purified by adsorption on controlled pore glass), 11 developed fever peaks exceeding 38 °C.

It is not known whether the fever reaction to leukocyte interferon is caused by the interferon itself or by impurities. In the case of fibroblast interferon, extensive purification using affinity chromatography on zinc chelate columns was able to remove all pyrogenicity detectable by intramuscular injection in man [27, 28]. Yet, intravenous infusion of this interferon which had a specific activity of more than 10^9 units/ml protein, still caused some fever after intravenous infusion. Thus, in all probability, fever after intramuscular injection of interferon is largely due to impurities.

Fever is a constant symptom occurring during interferon induction by viruses, endotoxins or synthetic inducers [29-31]. This allows speculation that interferon itself may be pyrogenic and may be responsible for fever and malaise during the acute phase of virus infection. The data obtained with pure fibroblast interferon would rather indicate that fibroblast interferon is at most a very weak pyrogen and that fever and malaise are due to factors co-induced with interferon. It may therefore be important to define and characterize the pyrogenic impurities in current interferon preparations.

Skin reactivity. Intracutaneous injection of fibroblast interferon preparations ($> 20,000$ units in 0.1 ml) was reported to provoke painless erythema and induration at the site of injection [4, 26]. This reaction occurred in patients who had never received any interferon before as well as in patients under continuous interferon therapy. The size of the reaction (average diameter with 20,000 units, 1 to 2 cm) varied from one test person to another but was remarkably constant when tested at different time intervals in a single individual. Patients with spontaneous or iatrogenic immunosuppression reacted as strongly as patients with a normal immune system. Pathologically the reaction resembled a delayed type hypersensitivity response, being characterized by perivascular mononuclear infiltrate, few polynuclear cells, very little oedema and absence of vascular lesions.

From experiments with completely pure fibroblast interferon [27, 28] it is now evident that this reaction is not caused by the interferon but by substances which, in all probability, are co-produced with interferon and were not removed by earlier partial purification methods. Again, it may eventually be important to define and characterize these skin reactive factors in fibroblast interferon preparations as they may throw new light on inflammatory responses seen during virus infections.

Cytostatic effects. *In vitro*, interferons have been shown to inhibit cell division in general and myeloproliferation in particular. Therefore, one might expect interferon therapy to be accompanied by some of the side effects of the chemical cytostatics used in cancer therapy. A mild myelosuppressive effect, as evident from peripheral blood cell counts, was sometimes seen in patients treated with leukocyte interferon for several days to weeks [25]. With fibroblast interferon no evidence for myelosuppression has been reported, except for an occasional patient who showed severe myelosuppression with leukocyte interferon and mild myelosuppression with fibroblast interferon [11, 26].

Recently hair loss (a classical side effect of therapy with chemical cytostatics) has been reported in 6 out of 37 patients treated daily with high doses of leukocyte interferon [32]. The same authors also reported elevation of serum transaminases in 62% of their patients.

Allergic reactions. Virtually all clinical studies up to this date were done with preparations of leukocyte and fibroblast interferon that contained various proteins of non-human origin: Sendai virus and egg proteins in leukocyte interferon and calf serum proteins in fibroblast interferon preparations. It is therefore surprising that no mention has been made of allergic reactions in patients treated for several weeks with these preparations.

Patients treated with fibroblast interferon from our laboratory were monitored for the development of allergy by regular skin testing (intracutaneous injection of 20,000 units/0.1 ml of interferon). The majority of patients showed no reactivity other than that observed in virgin patients [4]. Occasion-

ally, a patient developed severe reactivity [28] as manifested by a large wheel and flare reaction upon skin testing. When these patients were subsequently given skin tests with highly pure interferon ($> 10^9$ units/mg protein) no reaction ensued, indicating that sensitization was not directed to interferon and that the purification method (adsorption to controlled pore glass + zinc chelate chromatography) effectively removed foreign allergenic proteins.

In conclusion, it seems reasonable to assume that many of the side effects (fever, skin reactivity, allergy) seen during interferon therapy are due to impurities still present in current preparations. Whether this is also true for the cytostatic effects is less clear. Much of this confusion would be resolved if one could use completely pure interferon stabilized with inert protein such as human serum albumin. The methods for obtaining such preparations are now available [27, 33]. The use of such clean interferon preparations is even more recommendable since the number of patients given experimental interferon therapy is now steadily increasing and since, therefore, the chances for severe allergic accidents are also increasing.

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LARGE-SCALE PRODUCTION AND PURIFICATION OF HUMAN LYMPHOBLASTOID INTERFERON*

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The production of human interferon is of great importance, since this material is now used in controlled clinical studies. In order to meet the quickly growing demand for human interferon, we have developed a method, which can be scaled up to give large amounts of this substance.

The cells chosen for interferon production are "Namalwa" cells, a B-type lymphoid line transformed by an incomplete Epstein-Barr virus genome, established by KLEIN *et al.* in 1972 [1]. This line has been shown to give high yields of interferon after infection with Sendai virus [2] and is widely used by many investigators. The interferon produced after induction with Sendai virus closely resembles human leukocyte interferon, a minor fraction also showing the specificity of human fibroblast interferon [3].

The method differs in some respects from the methods used by others.

1. Human serum is used for cell culture. This prevents any contamination of the purified interferon with non-human serum proteins.

2. Sendai virus preparations used for infection are purified by high-speed centrifugation, in order to remove soluble egg proteins. Excess virus is removed by centrifugation.

3. No serum is added at the phase of interferon formation, resulting in high specific activity of the crude interferon.

The purification procedure adopted by us is composed of steps already known to workers in this field. The method is rapid and batches of up to 100 litres can be handled in a laboratory. It should provide interferon potent and pure enough for controlled clinical trials.

Cell culture and interferon induction

"Namalwa" cells, clone 8, were a kind gift of G. KLEIN, Karolinska Institute, Stockholm. They are grown in a medium based on RPMI 1640. To 100 litres of this medium are added 10–20 litres of Difco tryptose phosphate broth and 2 litres of polyethyleneglycol treated human serum.

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This serum is prepared by the method of INGLOT *et al.* [4]: polyethylene-glycol 6000 is added to inactivated human serum to a final concentration of 6%, the precipitate is removed, and the clear supernatant is used for cell culture.

Cells are grown in suspension culture, using an 80 litre air-lift type fermentor. Under optimal conditions (temperature 36.5 °C, pH 7.0–7.2), cells have a doubling time of 24–30 h. Starting at a cell density of about 0.7×10^6 cells/ml, densities of $2.5\text{--}4.0 \times 10^6$ cells/ml can be reached in 3–4 days of culture. Plateau-phase cells are then collected using a continuous flow centrifuge, and the cells resuspended in fresh medium at a density of $50\text{--}100 \times 10^6$ cells/ml. Sendai virus is then added to a concentration of 2^{10} haemagglutinating units/ml and the mixture is shaken for 1–2 h at 36 °C. The infected cells are again centrifuged to remove excess virus and resuspended at 5×10^6 cells/ml in Eagle's Minimum Essential Medium (MEM) without serum. After incubation at 35 °C for 20 h, crude interferon is obtained after removal of cellular debris by centrifugation. In order to inactivate residual virus, the crude interferon is cooled to 10 °C and adjusted to pH 2.0. It is then stored at 4 °C until used for purification.

Table I

Purification and controls of starting materials for production of namalva-interferon

Human serum for cell culture

1. Tested for absence of HBs-Ag by radioimmune-assay
2. Treated at 56 °C for 1 h
3. Treated with 6% w/v polyethylene-glycol 6000, precipitate discarded

Sendai virus

1. Purified by one cycle of low- and high-speed centrifugation
2. Tested for sterility
3. Tested for HA-content (min. 2^{11} HA-units/ml)

Cell culture

1. Daily microscopic inspection of cell growth and absence of contamination
2. Samples tested monthly by specialized laboratory for contamination by bacteria and mycoplasma

Table I summarizes our efforts to prepare and control the materials used for interferon production. The use of polyethyleneglycol treated serum not only improves its properties for cell culture, but also adds to the safety of the material, since many viruses which may contaminate the pooled serum, are precipitated along with the high molecular weight fraction and lipid-containing material of the inactivated serum.

Purification procedure

Concentration of crude interferon. The crude material is conveniently concentrated by the zinc acetate method originally described by LAMPSON *et al.* [5] for chicken interferon. Zinc acetate is added to a final concentration of 0.02 M and the pH adjusted to 7.2. The precipitate, which settles very quickly, is collected. It is dissolved again in ice-cold 0.1 M HCl using a suitable homogenizer to give a turbid solution of pH 2.5. The solution is then cleared by centrifugation.

Chromatography on SP-Sephadex C-25. The strongly acid ion exchanger-Sulfopropyl-Sephadex C-25 (Pharmacia) is used to adsorb the dissolved interferon. The necessary amount should be determined for each batch. Usually, 1.5 g of the material is sufficient for one litre of crude starting material. After stirring overnight at 4 °C, the ion exchanger is removed by settling and washed at pH 2.5 (0.1 M glycine/HCl) and at pH 4.0 (0.1 M K citrate buffer containing 0.005 M sodium versenate). It is then filled into a suitable chromatographic column with the pH 4 buffer. Interferon is eluted using 0.1 M K phosphate pH 8.0 containing 1 M KCl. The eluate is monitored for its extinction at 280 nm, and protein containing fractions are pooled.

Concentration with KSCN. The use of acid KSCN for the concentration of human leukocyte interferon was recommended by K. FANTES (personal communication) and is routinely used for the purpose. The SP-Sephadex eluate is adjusted to pH 3.5, the solution cooled in an ice-bath, and solid KSCN is added to give a concentration of 1 M. The precipitate, which forms quickly, contains the interferon and is collected by centrifugation. It is carefully drained and treated with 0.1 M K phosphate pH 8 to give a 3000-fold interferon concentrate of neutral pH. The turbid solution is cleared by high-speed centrifugation.

Gel filtration. In order to increase the safety of interferon preparations intended for clinical use, high molecular weight components should be removed, since any contaminating potentially harmful nucleic acids are of high molecular weight. We have, therefore, introduced a gel filtration step into the procedure.

Columns of Sephadex G-100 (Pharmacia) or Ultrogel AcA 54 (LKB), 1 litre in volume have been found equally effective. The eluant is phosphate buffered saline (PBS) pH 7.4. Columns are calibrated with proteins of known molecular weight before use. This greatly facilitates location of the interferon in the eluate (Fig. 1).

This procedure removes a substantial amount of high molecular weight material. Interferon is eluted as a rather symmetrical peak, corresponding to a molecular weight of $22\,000 \pm 4000$ daltons. For preparative purposes, eluate fractions corresponding to molecular weights from 15 000 to 35 000 daltons are collected and immediately processed. This eliminates the need for keeping the

eluate until interferon tests can be performed. The extinction profile at 280 nm is also of some help in finding the interferon: it usually coincides with a minor peak near 25 000 daltons. In any case, standing of the eluate should be avoided since losses tend to occur.

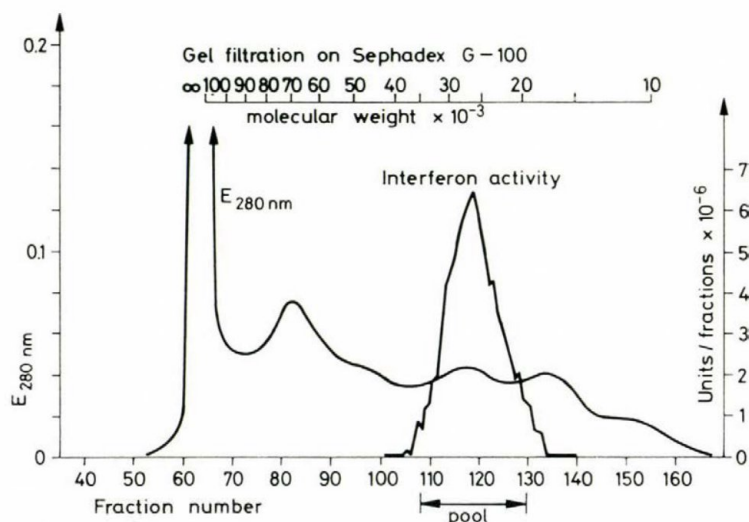


Fig. 1. Gel filtration on Sephadex G-100. Column size: 5 $\times$ 50 cm. Eluant: PBS at 40 ml/h 1 fraction = 10 min. Material applied to column: 130×10^6 units of interferon 152 mg protein. Molecular weight markers: bovine serum albumin, 69 000; ovalbumin, 44 000; horse myoglobin, 17 000

Concentration on SP-Sephadex C-25. Since gel filtration dilutes the interferon about 20-fold, concentration is again needed. A small (1 $\times$ 5 cm) column of SP-Sephadex is used for this purpose. The eluate is acidified to pH 3.0 and passed over the column which has been equilibrated with 0.1 M K citrate buffer pH 3.0. After a short wash, interferon is eluted with 0.1 M K phosphate pH 8.0. The protein containing fractions are pooled, dialyzed against PBS and the purified interferon is stored at -20°C .

Purification of a representative batch of crude interferon is shown in Table II. Titres of the crude material range from 1000–6000 IU/ml, with a mean of about 3000.

Recently, methods have been described to raise interferon yields by treatment of Namalwa cells before harvest with substances causing an arrest of DNA synthesis: bromodeoxyuridine [6], n-butyrate [7, 8], or glucocorticoid hormones [9]. In some preliminary experiments, interferon titres in crude harvests could be raised 2–5 times over the figure given in Table II. Such treatments are, however, always toxic to cells and difficulties arise on adopting such procedures to large-scale fermentor cultures.

Table II*Purification of human lymphoblastoid interferon*

Purification step	Units		Specific activity, U/mg protein
	U/ml	total (%)	
1. Crude (pH 2 treated)	3 200	55×10^6 (100)	59.000
2. Zinc acetate and Sp-Sephadex C-25	239 000	50×10^6 (91)	368.000
3. KSCN concentration	11×10^6	38×10^6 (69)	639.000
4. Sephadex G-100 (fraction pool)	690 000	21×10^6 (38)	2.2×10^6
5. SP-Sephadex conc. and dialysis PBS	3.9×10^6	15×10^6 (27)	3.4×10^6

Our procedure consists of 3 steps primarily intended for concentration: zinc acetate, KSCN, and second SP-Sephadex step. The remaining 2 steps are responsible for the greatest part of the purification. The final preparations exhibit a specific activity of several million units/mg of protein. Total yields are from 25 to 30%. The greatest loss, about 50%, is observed at the gel filtration step. It is the same for both materials tested, Sephadex G-100 (medium) or Ultrogel AcA 54. The cause of the loss is not known.

Purity requirements and safety considerations for clinical use

The problem of safety testing of interferon from different sources has first been raised at the NIH-workshop organized by J. C. PETRICCIANI in October, 1979. Meanwhile, we have adopted a preliminary schedule for purity requirements and final container testing for preparations intended for use in cancer patients.

Each purified lot is checked according to the following criteria:

(1) *Specific activity.* Following the recommendations, specific activity should at least reach 10^6 IU/mg of protein. (Standard: B 69/19 human leukocyte interferon.)

Table III*Purity requirements of interferon concentrates for use in osteosarcoma trials*1. *Specific activity*

at least 10^6 interferon units/mg of protein

2. *Protein composition*

Sample containing 20 μ g protein analysed by SDS-PAGE (tubes 5×80 mm). No protein components of molecular weight above 70 000 daltons should be detected (stain: Coomassie blue)

3. *Absence of Sendai and egg proteins*

Ouchterlony diffusion test against rabbit antiserum prepared with crude Sendai virus

(2) *Contamination with high molecular weight components.* Twenty μg of protein is applied to a 5×80 mm glass tube and analysed by SDS-polyacrylamide gel electrophoresis by the method of WEBER and OSBORN [10]. After staining with Coomassie brilliant blue, no protein bands in the region above 70 000 daltons should be seen.

Figure 2 gives a typical example of such a run (using a higher amount of protein). The interferon activity migrates as a rather broad peak, corresponding to about 20 000 daltons. It is clear that at this level of purity none of the stained bands represents the interferon.

Experiments have shown that the method can pick up about 1 μg of protein in the high molecular weight region of the gel. Since 20 μg is applied,

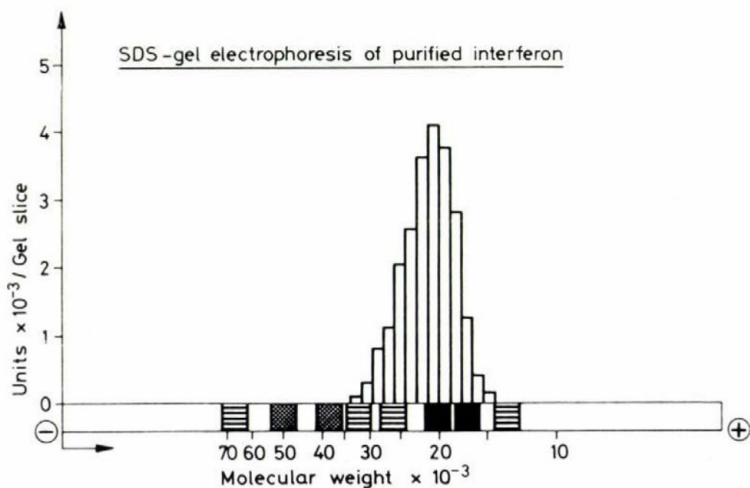


Fig. 2. SDS-gel electrophoresis of purified interferon. Material applied to one gel: (a) for protein staining 46 000 units (32 μg protein); (b) for interferon determination 115 000 units (80 μg protein). Molecular weight markers: bovine serum albumin 69 000; ovalbumin, 44 000; myoglobin (horse), 17 000

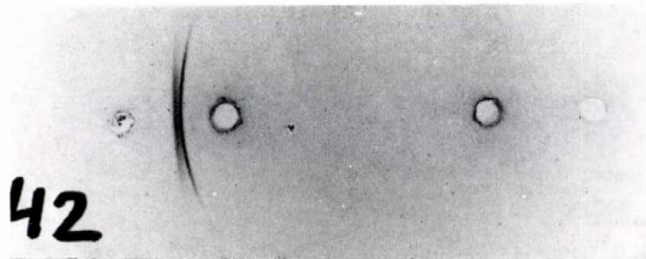


Fig. 3. Ouchterlony double diffusion of purified interferon against rabbit anti-Sendai globulin. To each hole, $2 \times 5 \mu\text{l}$ was applied. Microscope slide 26×76 mm. Left side: positive control. Outer hole: Sendai virus; inner hole: anti-Sendai globulin. Right side: interferon sample. Outer hole: interferon; inner hole: anti-Sendai globulin

a negative result indicates that less than 5% of total proteins could be of high molecular weight.

(3) *Contamination with Sendai and egg proteins.* A sample of 10 μ l (10–20 μ g of protein) is applied to a microscope slide coated with agarose in PBS and analysed by the Ouchterlony double diffusion technique [11], using globulin preparations from rabbits immunized with crude Sendai virus. No precipitation should occur with the interferon sample.

Using globulin preparations capable of demonstrating Sendai and egg proteins on diffusion against 10 μ l of a 1 : 100 dilution of crude Sendai virus (3–4 mg/ml of protein), the limit of detection of these proteins is less than 0.4 μ g. Since 10–20 μ g of protein is applied with the interferon samples, a negative result indicates that the amount of Sendai and egg protein is less than 4%. At the left side of Fig. 3 is the positive control. Diffusion of 10 μ l Sendai virus (outer hole) against anti-Sendai globulin (inner hole) gives a strong positive result. On the right side of the microscope slide 10 μ l of interferon (outer hole) is diffused against antiglobulin (inner hole). No precipitation can be seen.

Preparation of samples for clinical use

The interferon titre is adjusted to 1.5×10^6 units/ml, using PBS as diluent, and 2% human serum albumin (quality: "For intravenous injection") is added as stabilizer. The material is sterile filtered through a polycarbonate membrane filter of 0.2 μ pore diameter and dispensed in aliquots of 2 ml into suitable vials. These are kept at -20°C . At this temperature, the interferon samples are stable for at least one year.

A preliminary test schedule for the final containers has been adopted.

Table IV

Test schedule of interferon lots for clinical trials in osteosarcoma patients

Each lot must meet the following requirements:

1. *Interferon content of 1 ampoule*

3×10^6 units (min.: 2×10^6 , max.: 4×10^6); calculated mean of four consecutive tests

2. *Sterility*

According to the Austrian Pharmacopoeian Codex (ÖAB 9)

3. *General safety*

No detectable adverse reactions after i. v. injection of 5×10^5 units per 20 g mouse in 5 animals. (Body weight, general condition.)

4. *Absence of Sendai virus*

No formation of viral haemagglutinin after inoculation into embryonated chicken eggs

5. *Maximal pyrogenic reaction in rabbits*

I. v. application of 10^5 units/kg into 3 animals.

The sum of body temperature rise must not exceed 6°C

(1) *The interferon content* should be within 2 and 4×10^6 IU. The calculated content is 3×10^6 units (one dose).

(2) *Sterility testing* is performed according to the Austrian Pharmacopoea (No. 9).

(3) *General safety*. 5×10^5 units are injected intravenously into 20 g mice (5 animals). No adverse effects (weight, behaviour) should be noted for a period of 2 weeks.

(4) *Absence of live Sendai virus*. Aliquots of 0.1 ml are injected into the allantoic of 11-day-old chick embryos. After incubation at 36 °C for 48 h, no haemagglutinin should be found in the allantoic fluid.

(5) *Pyrogenic reaction in rabbits*. Three rabbits are given 10^5 units/kg of body weight. Pyrogenic reaction is recorded in the usual way. There has always been a temperature increase in these animals, usually of 0.5–1.5 °C. The sum of these in three animals should, however, not exceed 6 °C.

The fact that there is always some pyrogenicity, reduces the value of the test, which should show the absence of bacterial pyrogens in the preparations. Therefore, experiments using the limulus assay are planned.

Clinical application

At present, a small scale trial in osteosarcoma patients is under way. Hopefully, our supply of interferon will enable us to start some more small scale controlled clinical trials.

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GROWTH INHIBITORY EFFECT OF MOUSE INTERFERON IN TRANSFORMED CELLS*

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The long controversy about the non-antiviral activities of interferon (IF) in cells seems to be resolved, although not closed, in favour of accepting that the cell may respond in a number of ways to IF molecules [1, 2]. The main argument raised in support of such a conclusion is based on unsuccessful attempts to separate physically the molecules with antiviral and cell growth inhibitory activities in maximally purified preparations of IF [3, 4]. At the same time, additional evidence indicates that IF treatment alters the cell surface and leads to enhanced or depressed manifestations of various cell activities [5–8]. In contrast with the general sensitivity of animal viruses to IF, however, unpredictable variations among cells were observed in the cell growth inhibitory (CGI) test (Table I).

To gain more insight in the process that leads to such variations, in this study lines and clones of C3H mouse embryonic cells transformed by two different viruses and a carcinogenic chemical were compared in their reactivity to partially purified mouse IF. The strategy of our experiments is shown in Table II. (For technical details see BORECKÝ *et al.* [9]). In brief, mouse embryonic C3H fibroblasts in the second passage were exposed either to about 10^7 TCID₅₀ of SV40 or MSV (Harvey) virus, or, 0.1 µg per ml 20-methylcholanthrene (MCH). The viruses were left in contact with the cells for 4 h, while MCH was applied to cells for 72 h with daily exchange of medium containing the cancerogen. Foci of morphologically transformed cells appeared 4–6 weeks after seeding of virus infected cells and 3–4 weeks after seeding of cells treated with the carcinogenic chemical.

The cells from foci proved subculturable and initiated the transformed lines designated subsequently as MEF-SV40, MEF-MSV and MEF-MCH. These lines, in various passages, were tested for agglutinability with concanavalin A (Con-A), for growth characteristics (population density) in Eagle's basal medium supplemented with 20% of heated calf serum, for growth in

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Table I*Characteristics of the cell growth inhibitory effect of interferon**

1. Interferon preparations may cause both reversible and irreversible growth inhibitory as well as morphological and functional alterations in the cells.
2. The alterations are determined by species, tissue and type specificity of cells as well as physiological (passage history, age) and pathological (viral infection, oncological transformation) influences.
3. The cell growth inhibitory effect usually requires higher amounts of interferon and a longer contact with the cell than the antiviral effect. Cell populations usually contain a resistant fraction.
4. Cell populations, lines, or clones can be selected that are insensitive to the cell growth inhibitory effect while retaining the sensitivity to the antiviral effect of interferon.
5. The cell growth inhibitory effect can be separated from the antiviral effect by manipulation of cells.
6. Partial success was only achieved in this respect by manipulation of interferon preparations.

* Based on data reported by GRESSER [31, 32], GRESSER *et al.* [33], OHWAKI and KAWADE [34], BORECKÝ *et al.* [35, 36, 37], FUCHSBERGER *et al.* [13], KUWATA, *et al.* [2, 38], DAHL [15], GRESSER [1], KARANIEMI [39], HILFENHAUS (personal communication 1979).

Table II*Outline of tests of sensitivity of transformed mouse cells to the antiviral and cell growth inhibitory effects of interferon*

1. Mouse C3H fibroblasts (MEF) in secondary culture
2. Treatment with MSV (Harvey), SV40, and 20-methylcholanthrene
 - (a) Establishment of "transformed" lines (MEF-SV40, MEF-MSV, and MEF-MCH)
 - (b) Tests for agglutinability with concanavalin-A, growth in soft agar, interferon producing capacity, tumourigenicity, etc.
 - (c) Tests for sensitivity to cell growth inhibitory and antiviral effects of mouse interferon, etc.
3. Isolation of clones in soft agar
 - (a) Establishment of cloned lines and subclones
 - (b) Tests for binding of soybean lectin, concanavalin-A, phagocytosis by macrophages, and expression and topography of H-2.23 and/or H-2.32 specificities
 - (c) Tests as under 2/b and c

Table III*Characteristics of lines obtained after transformation of C3H mouse embryonic cells with SV40 or MSV viruses or 20-methylcholanthrene*

Cells (lines)	Passage <i>in vitro</i>	Agglutination with concanavalin A 100 µg/ml	Growth in semisolid agar	Population density per cm ² ($\times 10^3$)	Tumourigenicity in newborn C3H mice
MEF (Parent)	2	—	—	49	0/3
MEF-SV40	5	++	+	200	0/3 (18 p) ¹
MEF-SV40	21	ND	+	560	ND
MEF-MSV	15	+++	—	150	3/3 (17 p)
MEF-MSV	26	ND	—	510	6/6 (35 p)
MEF-MCH	9	++	—	100	0/3
MEF-MCU	32	ND	+	410	ND

¹ Passage number tested

ND = Not done

semisolid agar according to MAC PHERSON and MONTAGNIER [10], and for tumourigenicity in newborn syngeneic C3H mice.

As shown in Table III, in contradistinction to parental cells, the transformed lines were agglutinated by less than 100 μ g of Con-A and reached 2–10 times higher population densities than the parental strains. On the other hand,

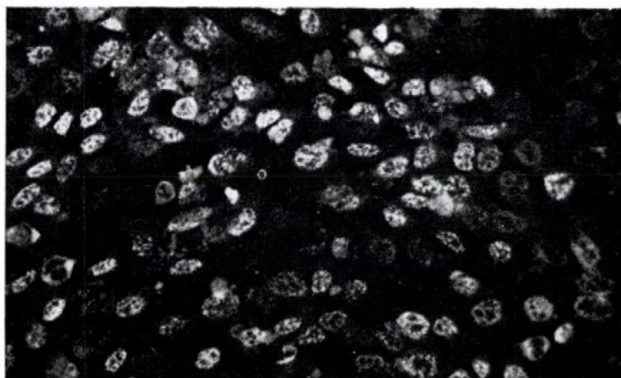


Fig.1. T-antigen in SV40 transformed cell line detected with immunofluorescence. $\times 400$

only the MSV-transformed line proved oncogenic for newborn C3H mice in passages tested but this line did not grow in semisolid agar.

A reverse situation was observed with the cells of the SV40 line which grow readily in semisolid agar (tested in the 8th and 21st passage) but were not tumourigenic for newborn C3H mice. The MCH line was non-tumourigenic in the 9 early passages and acquired the capacity to grow in semisolid agar during 30 passages *in vitro*. The SV40 cells were T-antigen positive by the immunofluorescence technique (Fig. 1). The chromosome number of the MSV line was 63 ± 3.3 while the chromosome number of the parent C3H cells was 38 ± 0.51 .

Interferon production and interferon sensitivity of transformed lines

As shown in Table IV, MSV-transformed cells retained their IF producing capacity but it was lost in the SV40-transformed cells. The MCH line behaved again as intermediary since it produced no detectable IF when stimulated with NDV in the 9th passage but regained this ability in later (35th) passages. The SV40-transformed cells differed from the other 2 lines in an enhanced resistance to EMC virus, which made the testing of the antiviral effect (AV) of IF in these cells often difficult. The MEF–MCH cells seem to be as sensitive as their parental cells to the AV effect of IF, while the MSV cells show a lowered sensitivity. Preliminary tests suggested a slightly enhanced resistance of

Table IV

Interferon producing capacity and interferon sensitivity of the MEF-SV40, MEF-MSV, and MEF-MCH cell lines in different passages

Cells (lines)	Passage in vitro	Production of IF (per ml) ¹	Sensitivity to	
			AV of IF (titre per ml)	Units for CGIE of IF (30% growth reduction)
MEF (Parent)	2	320	20 000	1500
MEF-MSV	8	320	ND	ND
MEF-MSV	12	320 ±	1 280	ND
MEF-MSV	20	ND	2 560	750 (22nd passage)
MEF MSV	28	640	ND	ND
MEF-SV40	14	10	ND	3000 (7th passage)
MEF-SV40	25	10	160 000 ²	ND
MEF-SV40	37	ND	ND	ND
MEF-MCH	9	10	ND	ND
	22	ND	40 000 ±	750 (19th passage)
	35	80	ND	ND

¹ Tested in L-929 cells against EMC virus

² The MEF-SV40 line showed a diminished sensitivity to the CPE of EMC virus. The effective amount of the virus in IF tests was 1 to 10 CPD₅₀ in SV40-transformed cells and 100 CPD₅₀ in other tested cell lines

ND = Not done

MEF-SV40 cells to the cell growth inhibitory effect (CGIE) of IF. The sensitivity of MEF-MCH and MEF-MSV cells to the CGIE of mouse IF was about two times of that of SV40-transformed cells.

Properties of clones isolated from transformed MEF-MCH and MEF-SV40 lines

In the 8th passage of MEF-SV40 line 8 clones were isolated from colonies growing in semisolid agar for 12–15 days by the technique of MAC PHERSON and MONTAGNIER [10]. These clones as well as some subclones were further analysed for sensitivity to AV and CGIE of IF in various passages and sub-passages (Table V). The results indicated that (a) the SV40 clones, like the parental SV40 line, do not produce interferon on NDV stimulation; (b) show a relatively high AV sensitivity; (c) can be divided into 3 groups with regard to sensitivity to the CGIE of IF; (d) the subclones seem to retain the relative resistance to EMC virus infection (Table VI).

The MEF-MCH clones were started after the cells in the parental line had acquired the capacity to grow in semisolid agar, i.e. after 30 passages.

Table V*Interferon sensitivity and interferon production of SV40-transformed clones*

Group	Clone No.	Passage <i>in vitro</i>	CGI titre ¹	AV titre ² × 10 ³	IF production ³
I	5	18	24	160	< 10
	27	18	110	160	< 10
	35	18	50	80 ±	< 10
	35-4 ⁴	39 (+3)	50	80	ND
	35-8 ⁴	39	50	80	ND
II	3	39	2200	640	< 10
	30	39	1540	20	< 10
	44	39	1400	320	< 10
III	31	39	3000	320	< 10
	63	39	3000	320 ±	< 10

¹ Interferon units required for a 30% inhibition of multiplication² Highest dilution of interferon inhibiting the CPE of 100 TCID₅₀ of EMC virus. (In tests with clones No. 35 and No. 30 the effective number of TCID₅₀ was more than 100)³ Antiviral titre in the supernatants of cells stimulated with Newcastle disease virus⁴ Subclone — In parenthesis, the number of passages of the subclone

ND = Not done

Table VI*Cytopathic titres of encephalomyocarditis (EMC) virus in various clones of SV40 transformed lines*

Clones of MEF-SV40 cells used for EMC virus titra- tion	Range of CPE titre in repeated tests
No. 3	10 ⁻² –10 ⁻⁵
5	10 ⁻² –10 ⁻⁴
27	10 ⁻² –10 ⁻³
30	10 ⁻² –10 ⁻⁵
31	10 ⁻² –10 ⁻⁴
35	10 ⁻² –10 ⁻⁵
44	10 ⁻² –10 ⁻⁵
53	10 ⁻² –10 ⁻⁵
MEF-MSV (line)	10 ⁻⁶ –10 ⁻⁷
MEF-MCH (line)	10 ⁻⁶ –10 ⁻⁷
Control (C3H)	10 ^{-6.5} –10 ^{-7.1}

Table VII

Sensitivity to interferon of 20-methylcholanthrene transformed clones and their interferon producing capacity

Group MEF-MCH	Clone No.	Passage	GCl titre ¹	AV titre ² × 10 ³	IF production ³
I	36	20	640	64	80 ±
	43	11	400	128 ±	80
	49	16	700	128	80 ±
	52	17	580	64	160 ±
II	41	11	1300	64	80
	57	22	2500	64	80
	57/5 ⁴	32 (+2)	2500	80	ND
	73	12	2500	128	160 ±

¹⁻⁴ For explanations, see Table V

Table VIII

Chromosome numbers of transformed mouse cell lines and clones

Cells (lines, clones and subclones)	Passage	Number of chromosomes ³	
		Range	Mean ± 1 SD
MEF (parent)	2	36-40	38 ± 0.51
MEF-MSV (line)	29	57-66	63 ± 3.3
MEF-SV40-35 (clone)	23	40-54	46 ± 3.1
MEF-SV40-35-4	3	40-46	43 ± 1.7
MEF-MCH 36 (clone)	6	80-94	87 ± 4.6
MEF-MCH	20	76-95	86 ± 4.4
MEF-MCH 36/1 ²	4	73-96	85 ± 4.8
MEF-MCH 36/2 ²	3	76-97	86 ± 4.5
MEF-MCH 49	17	84-104	93 ± 6.7
MEF-MCH 49/1 ²	1	80-106	94 ± 6.1
MEF-MCH 52	9	85-104	95 ± 5.1
MEF-MCH	18	72-96	87 ± 4.9
MEF-MCH 57	29	78-92	85 ± 4.15
MEF-MCH 57-5'	2	72-92	85 ± 4.2

¹ Subclone of SV40-35 or MCH 57 clone

² Cells from tumours induced in mice with clones 46 or 49 after one (36/1, 49/1) or two (36/2) additional passages *in vitro*

³ Number of analysed mitoses, 30

These clones (a) show a relatively uniform IF producing capacity; (b) did not differ significantly in sensitivity to the AV effect of IF; (c) can be divided into 2 groups with regard to sensitivity to CGIE of IF; and (d) the subclones behave like the parental clone (Table VII).

The chromosome number of tested clones is shown in Table VIII. It indicates a weaker deviation from the parental MEF cells in the case of MSV-transformed cells than in the case of MCH-transformed clones. The chromosome-numbers, however, showed no correlation with other parameters (Tables II and III). For instance, the MCH clone No. 52 had 95 ± 5.1 chromosomes and was non-oncogenic for newborn C3H mice while clone No. 36 and its subclone 36/1 had 86 ± 4.4 and 85 ± 4.8 chromosomes, respectively, and was oncogenic.

Since attempts to grow MSV transformed cells in semisolid agar were unsuccessful, no clones were available for comparative tests.

Surface properties of cloned cells

It is now well established that IF alters the cell surface [1, 11, 12]. Such alterations are presumably a prerequisite for various manifestations of the IF-effect in the cell. In order to gain some insight in the surface differences among cells, clones differing in sensitivity to the CGIE of IF were examined for (a) their binding capacity of two lectins (soybean lectin and Con-A); (b) expression of H-2 specificities on their surface; and (c) sensitivity to macrophages. As shown in Table IX, a certain correlation of sensitivity to IF and Con-A was observed in the MCH clones in accordance with results of KUWATA *et al.* (to be published). The correlation was inverse when soybean lectin was used as reagent (Fig. 2).

A positive correlation seems to exist also between the sensitivity of the clones to CGIE of IF and the cytostatic effect of macrophages (Fig. 3). The

Table IX

Cell growth inhibitory effect of interferon and concanavalin-A in MEF-MCH clones

Clones of MEF-MCH cells	Number of cells in per cent of control after treatment* with	
	5000 IF units	50 μ g Con-A
No. 36	45	85
52	40	75
57	75	106
73	75	110

* The number of cells was estimated on the 4th day after explantation and 3 days after IF or concanavalin treatment.

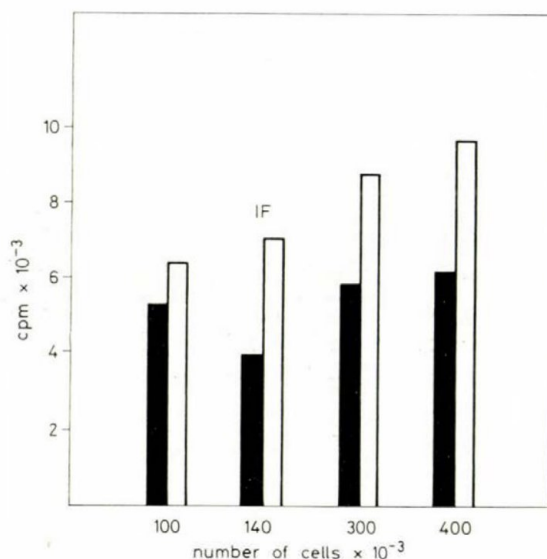


Fig. 2. Binding of ^{125}I -labelled soybean lectin to interferon sensitive (No. 36) and resistant (No. 57) MCH clones. Open columns: clone No. 57; closed columns: clone No. 36. Ordinate: $\text{cpm} \times 10^{-3}$. Abscissa: number of cells $\times 10^{-3}$ treated with lectin

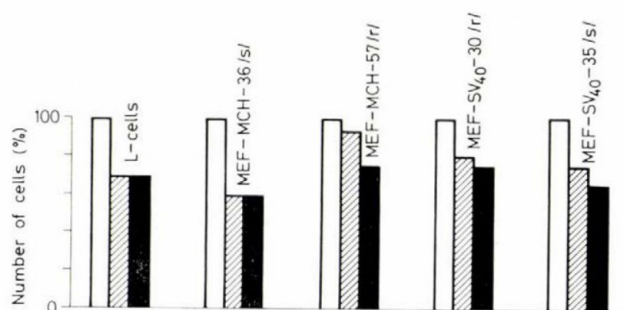


Fig. 3. Growth inhibition of MCH and SV40 transformed clones by activated mouse peritoneal macrophages. Open columns: control cells; shaded columns: ds-RNA activated cells; closed columns: IF activated cells. Ordinate: number of cells in per cents of non-treated cells. Mouse peritoneal macrophages were activated with phage f2 ds-RNA ($10 \mu\text{g}$ per 2×10^6 cells at 37°C for 2 h), or 100 units per ml of interferon. The number of surviving target cells was estimated in 3 parallel experiments, after 72 h of contact with macrophages

tested MCH clones No. 36 and 57, also differ in expressing some H-2 specificities. This relates especially to CGIE sensitive clone No. 36. Interferon markedly enhanced the antigen expression in the resistant clone No. 57 (Fig. 4).

As shown in Fig. 5, IF enhanced the agglomeration of those H-2 antigens which were coded by the K-region, while had no (clone No. 36), or an opposite effect on H-2 antigens coded by both terminal regions K and D (clone No. 57).

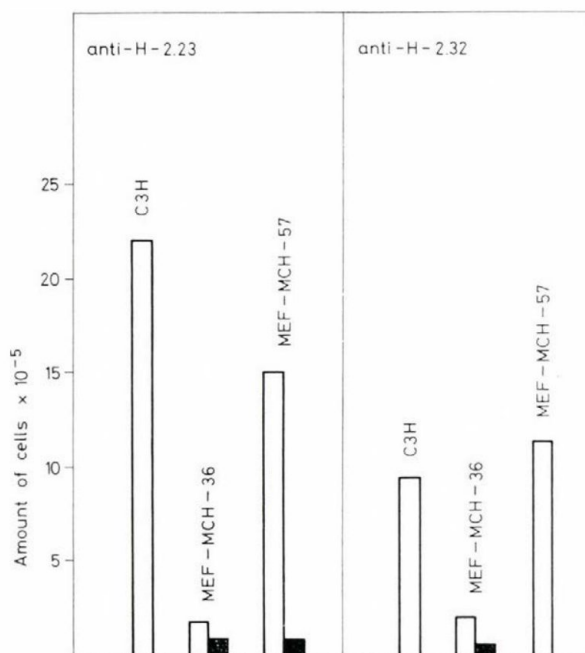


Fig. 4. Residual cytotoxic activity of anti-H-2.23 (anti-k) and anti-H-2.32 (anti-d) sera for ^{51}Cr -labelled C3H cells after absorption with interferon treated MCH-36 or MCH-57 cells. Shaded columns: IF treated cells; open columns: non treated cells. Ordinate: number of cells $\times 10^{-5}$ required for a 50% reduction of cytotoxicity of the sera

This follows from results of testing the "blocking index" [13] of specific antik- and anti-d sera in control and/or IF-treated MCH 36 and MCH 57 cells differing in sensitivity to the CGI effect of IF.

Discussion

Several reports indicate that the CGI and AV effects of IF are not necessarily parallel and are separable. Isolation of cell lines with divergent sensitivity to the AV and CGI effects of IF lends credit to such a conclusion [2, 14, 15]. Since virus multiplication involves various functions of the cell machinery, it is conceivable that their inhibition by IF may contribute in several ways to a suppression of virus-multiplication [16]. This fact shifts the problem of IF action on the cell from the realm of virology to that of colicins, chalones and polypeptide hormones [17-19]. In view of the fact that cyclic nucleotides seem to be involved in various hormonal effects in animal cells [20], these substances are candidates for mediating the various effects of IF [21]. Supposedly, IF activates a membrane adenylase which raises the level of cyclic AMP in the cell [11]. Cyclic nucleotides, however, were not effective in enhancing the

CGIE of IF in the resistant CSV 21 subline on LB2 cells [22]. Moreover, if cyclic nucleotides are the mediators of IF action, one would expect that they will, after addition to IF sensitive cells, mimic its action. This, however, has not been shown. On the other hand, it cannot be excluded that "second messengers" of some unknown type may participate in the CGIE of IF. Interestingly, no experiments have been published reporting transfer of CGIE from IF treated to non-treated cells while this was clearly demonstrated in the case of AV effect [23].

In this study, three lines of cell transformed by 3 carcinogenic agents but originating in one parental population were used for comparative tests.

The availability of parental "normal" cells allowed to follow up the behavioural changes of transformed cells during passages *in vitro* and to obtain insight in the effect of IF in cloned tumour cells. The results support the view that the AV and CGI effects are cell-dependent manifestations of IF action and that transformation of cells with different carcinogens in a different way influences such manifestations. This was documented by finding that neither the SV40 virus transformed cell line nor the clones isolated from this line produced IF on stimulation with Newcastle disease virus while the MSV transformed cells were not altered in this capacity. Interestingly, the transformation by MCH proved to be a two-stage process requiring cells that acquired the capacity for an anchorage independent growth [24]. Concurrently, their IF producing capacity was also recovered (Table III).

Non correlation between the two activities of IF were found in cloned lines after transformation with SV40 virus or the carcinogen 20-methylcholanthrene. Moreover, the various clones isolated from the same line differed in sensitivity to CGIE of IF, while showing a uniform behaviour in AV tests. This suggests that surface differences could play a decisive role in the CGIE of IF while they seem less significant in the AV effect.

Although the character of surface components (receptors) determining the IF binding has not been identified, different receptors or different numbers of receptors may be required for both effects. This view is supported by finding a negative correlation between soybean-lecting binding and a positive correlation between Con-A binding on the one side and CGI sensitivity of the clone on the other. On the other hand, the differences between AV and CGIE could be quantitative. As shown in Fig. 5, IF diminished the distance between glycoprotein antigens coded by the K-region. This suggests that a similar mechanism might be operative in other IF effects. While a co-operation of receptors might be required for CGIE, such a co-operation does not seem to be necessary for the AV effect. Such a possibility is supported by the fact that in the majority of cell systems, greater amounts of IF, longer contact, and, exponentially growing cells were required for demonstrating a CGIE (Table I). In this sense, the AV effect of IF might represent only a highly sensitive side

effect of cellular reactions initiated by IF. Alternatively, in analogy with some suggested hormone mechanisms the various effects of IF could reflect the various extent of cell surface perturbations which follow the action of IF on the cell [6, 25].

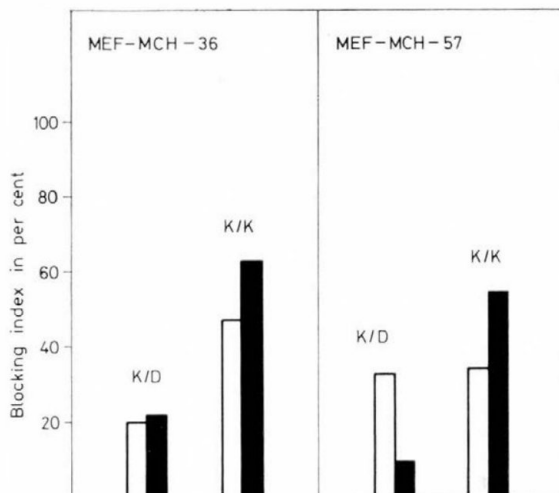


Fig. 5. Topographical relations between H-2 antigens on interferon treated and control MCH-36 and MCH-57 cells expressed by the "blocking index" [13]. Open columns: control cells; closed columns: interferon treated cells. K, D: H-2 coding regions. Ordinate: blocking index in per cent. Abscissa: antigenic specificities coded by one (K/K) or two (K and D) regions

The CGIE sensitive and less sensitive clones differed also in sensitivity to the cytostatic effect of mouse peritoneal macrophages. This supports the observation of IMANISHI *et al.* [26] that the target cell may also be favourably modified by IF for subsequent phagocytosis.

In several respects, the CGIE of IF resembles the effect of chalcones. Several reports indicated that IF is more active in aged than in young cells [27, 28]. Recently, this has been clearly demonstrated also for chalcones [29]. In addition, growth factors in the case of chalcones, and, antagonists were shown to regulate the IF effect [30].

In summary, although the mechanism of the CGIE of IF remains unclear, the CGIE is firmly established and classifies IF as a regulatory substance with a broad cellular activity. The present study indicates that the regulatory effect of IF shows clonal distribution and the sensitivity of cells to IF cannot be predicted without previous testing.

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SOME RECENT ADVANCES IN THE MOLECULAR BIOLOGY OF THE INTERFERON SYSTEM*

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Research on the molecular biology of the interferon system is flourishing. This is partly because of the recent surge of interest in the possible use of interferon in cancer patients, with a resultant flow of money and people into the field. However, at a more basic level, it is due to an increasing interaction with modern cell and molecular biology — which on the one hand can provide interesting new tools to investigate the interferon system, while on the other, the interferon system itself provides an interesting system to study the control of gene expression. In this brief review I propose to review three new areas of interferon research: the use of somatic cell hybrids, of gene cloning and the preparation of a monoclonal antibody to interferon.

Somatic cell hybrids in interferon research

When cells of two species, say mouse and human, are fused by use of Sendai virus or polyethylene glycol, a heterokaryon is produced, containing two distinct nuclei and common cytoplasm (see RINGERTZ and SAVAGE [1] for a review). At mitosis, the two nuclei fuse to produce two daughter cells, each of which has a single nucleus containing both human and the mouse chromosomes from the parents. Upon subsequent cell division the chromosomes of one parent are gradually lost, and in the case of hybrids formed between mouse cell lines and human cell strains the human chromosomes are lost. Loss is initially rapid, but the chromosomal contents then stabilize to produce a hybrid cell containing a complete set of the mouse chromosomes but only some human chromosomes. Different clones of cells contain different sets of human chromosomes, for the process seems to be largely random, and these clones can be grown up and their content of human chromosomes determined — either by karyotyping or by isozyme analysis. The hybrids can then be used to determine the chromosomal site of the interferon gene in the following ways:

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(a) The hybrids can be screened for a particular biological function — in this case the capacity to produce interferon after treatment with a virus or double-stranded RNA. Clearly, the only hybrids that will produce interferon are those that contain the chromosome that carries the interferon gene, and it may be possible, by comparing the human chromosome composition and the interferon producing capacity, to identify by inspection the human chromosome responsible.

(b) Hybrids continue to lose human chromosomes spontaneously and so by picking subclones from a particular hybrid, it is often possible to correlate a change in phenotype with loss of a particular chromosome.

(c) Hybrids can also be placed under pressure to lose a particular chromosome, and this makes it possible specifically to eliminate a chromosome that is suspected of carrying the gene in question — to determine whether there is the predicted change in phenotype.

The method, although straight forward in principle, has a number of drawbacks. Chromosomes can fragment, so that they are unrecognizable by karyotype analysis, or may translocate onto another chromosome. In addition, cultures of hybrids are often not homogeneous and so the interferon may be produced by a minority of the cells in the culture, while the karyotype is that of the majority of these cells. In such cases a false conclusion will be drawn as to the location of the gene. Finally there may be more than one gene involved, either because the gene product is, for example, coded by one gene but modified by the product of another or because the gene may be duplicated and copies present on different chromosomes.

The gene responsible for interferon action has been shown to be on human chromosome 21 by a number of workers (see STEWART [2] for a review). Locating the gene(s) responsible for interferon production has been more complicated. TAN *et al.* [3] made the first assignment to human chromosomes 2 and 5 — concluding that both chromosomes were necessary — while MORGAN and FAIK [4], using only a single hybrid for analysis, concluded that only chromosome 5 was necessary. Finally SLATE and RUDDLE [5], in work published after our own was completed, produced evidence for chromosomes 2 or 5.

In our own work a series of mouse-human hybrids were made in this laboratory and characterized by karyotyping by Dr. A. MEAGER *et al.* [6] and by isozyme analysis by Dr. D. SWALLOW in the Human Biochemical Genetics Unit, London. Other mouse-human hybrids and some Chinese hamster-human hybrids were obtained from colleagues. When the hybrids were induced to make interferon, a number of them made human interferon, while they all, as expected made mouse interferon. The hybrids which made human interferon, however, contained so many human chromosomes that it was impossible to identify which chromosome carried the gene for human interferon. Isolation of a number of clones, however, showed that the capacity to make

human interferon was lost whenever human chromosome 9 was lost, but that there was no loss of interferon production when chromosome 5 was lost. It was tentatively concluded that human chromosome 9 carried the interferon gene. Further information was sought by eliminating specific chromosomes: chromosome 5 was lost after cloning in medium containing diphtheria toxin since this chromosome carries the receptor that codes for the receptor for diphtheria toxin, and chromosome 2 was eliminated as a candidate by using a series of Chinese hamster-human hybrids that do not contain chromosome 2 but do make interferon. Finally a series of hybrids were made in which the human parent contains an X : 9 translocation, so that chromosomes X and 9 are fused. Although it is not possible to select for or against chromosome 9, it is possible to select for or against chromosome X and in the hybrid with the translocation, this selects for or against chromosome 9 as well. These studies showed conclusively that human chromosome 9 carried the interferon gene [7], and this was confirmed when it was found that a mouse-human hybrid which contained only a single human chromosome — number 9 — produced human interferon. Human chromosome 9 is therefore necessary and sufficient.

How then can we explain results that suggest chromosomes 2 or 5 carry an interferon gene? There are, of course, two types of human interferon — leukocyte or α interferon, and fibroblast, or β interferon. Are different workers looking at different interferons? All the interferons made by hybrids to date are fibroblast, and it is that gene which is on 9 [6] or on 2 or 5 [5]. Possibly there is more than one fibroblast gene, as there are leukocyte genes, and they are on separate chromosomes.

We have also examined the control of interferon production in these hybrids — looking at priming, superproduction and glycosylation [8]. In each case the human gene behaved as if it were under mouse control, and we have no evidence for any non-structural human genes that modulate the interferon response, although we could not exclude the possibility of such gene(s) on the same chromosome as the structural gene. Further analysis will use cloned genes to identify the precise location and arrangement of the interferon genes.

Gene cloning in interferon research

Recent advances have made possible the cloning of a number of eukaryotic genes in bacteria [9]. Such techniques make possible the preparation of large amounts of the gene, providing sufficient material for sequence analysis, and for use as a hybridization probe. The method starts from messenger RNA (mRNA) which is converted enzymatically to DNA (called cDNA). This cDNA is then inserted into a bacterial plasmid and then into a bacterium. The presence of such an inserted DNA sequence is then tested for — usually using a nucleic acid hybridization procedure. Once the bacterial clone that carries the

particular gene has been isolated, growth of the bacteria provides large amounts of the inserted DNA, and if the inserted DNA is positioned correctly in the plasmid in relation to promoters and ribosome attachment sites, then the gene can be translated to yield the eukaryotic protein.

This technique offers two advantages for interferon research. First it provides a method for preparing large amounts of the interferon gene which can then be sequenced. Second, if translation can be achieved, then bacteria might provide a much cheaper source of human interferon than using human cells. However, although attractive, this research is not easy and there are two main problems. The first is that the interferon mRNA represents only a small proportion of the total isolated mRNA and therefore most of the bacteria that contain inserted cDNA will contain cDNA to the other mRNAs that are present. This means that most of the clones — probably more than 99% — will have nothing to do with interferon. Therefore large numbers of bacterial colonies will have to be grown up and screened for the interferon cDNA insert. The second problem is that the screening method is technically very difficult. Each DNA to be tested needs to be hybridized with interferon mRNA, and then any DNA : RNA hybrid that is formed is isolated, melted to release the interferon mRNA which is then injected into *Xenopus* oocytes where it is translated to give human interferon, which after secretion from the oocyte, is assayed for its interferon content. It is easy to imagine how difficult and tedious a large number of such assays are.

It is therefore a very considerable achievement that human leukocyte [10] and fibroblast genes [11–13] have been cloned and the DNA has been sequenced [14, 15]. Both cloned genes have a signal sequence following the initiation triplet which is removed during processing of the polypeptide. This is followed by a coding sequence for 166 amino acids followed by a termination codon and an untranslated region. Comparison of the sequence of these two genes showed that there was 45% homology at the DNA level and 29% at the amino acid level [16]. The two genes are clearly, but not closely, related. The amino acid sequences are somewhat less related to each other than are the sequences of α and β globin and suggest that the two genes arose by a gene duplication, followed by evolutionary divergence, about 500 to 1000 million years ago. It has recently become clear that there are a number of leukocyte genes, presumably due to gene duplication, but it is not known yet how they differ from each other. Over the next year or two the structure of these cloned interferon genes will be investigated in detail, they will be used as hybridization probes to investigate some of the mechanisms involved in interferon control, and they will be developed as commercial sources of interferon.

Monoclonal antibody to leucocyte interferon

The normal immunological response to an antigen involves the production of several different types of antibody to a number of different antigenic sites. The antibody response is therefore polyclonal, that is the antibody producing cells are heterogeneous, being derived from a number of progenitor clones. The technique introduced by KOHLER and MILSTEIN [17] provides a way of immortalizing an antibody producing cell, making possible the isolation of a cell that grows in culture or in the mouse and that constantly produce a single antibody of defined structure and with reproducible activity. This antibody producing cell is a hybrid cell, derived from the spleen cell as one parent and a myeloma cell as the other. The spleen cell parent contributes the ability to make a specific antibody, and the myeloma cell the ability to grow in culture or in the mouse. In collaboration with Dr. DAVID SECHER of the Laboratory of Molecular Biology, Cambridge, we fused mouse myeloma cells with the spleen from a mouse that had been injected repeatedly with human lymphoblastoid interferon (in which leucocyte interferon is the major component), and that had a high circulating titre of antibody against interferon. The hybrid cells which were produced were screened for the capacity to release an antibody that neutralized leucocyte interferon, and a clone was isolated that secreted such an antibody [18]. This antibody, as the IgG, has been bound to Sepharose and used to purify crude interferon 5000 $\times$ in a single step, thus providing an attractive route for the large-scale purification of leucocyte interferon. In addition, the antibody is being developed as the basis of radioimmune assay for interferon, and as an international reference standard. The way now lies open to prepare monoclonal antibodies to fibroblast (β) and Type II (γ) interferon.

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NUCLEOSIDE ANALOGUES AS ANTIVIRAL AGENTS*

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Present state of the art

With the notable exception of interferon, amantadine and phosphonoformate, all chemicals that are currently used or considered for use as antiviral chemotherapeutics belong to the class of nucleoside analogues. This includes a number of compounds such as idoxuridine (IDU), trifluorothymidine (TFT) and adenine arabinoside (vidarabine, vira-A, Ara-A) which have been recognized as antiviral agents 15–20 years ago and which may therefore be referred to as first-generation antiviral drugs (Table I). In recent years several new nucleoside analogues have been developed or discovered which appear to surpass IDU, TFT and Ara-A in either potency or selectivity, or both. This second-generation of antiviral drugs encompasses thymine arabinoside (ara-T), 5-ethyl- and 5-propyl-2'-deoxyuridine (EDU, PDU), 5'-amino-5-iodo-2', 5'-di-deoxyuridine (AIDDU), acycloguanosine (acyclovir, ACG), (*E*)-5-(2-bromovinyl)-2'-deoxyuridine (BVDU), 2'-fluoro-5-iodo-aracytosine (FIAC), virazole (ribavirin) and (*S*)-9-(2,3-dihydroxypropyl)adenine [(*S*)-DHPA] (Table I).

The three first-generation drugs IDU, TFT and Ara-A (Fig. 1a, b, c), have all been licensed for clinical use: IDU (as 0.1% eye drops), TFT (as 1% eye drops) and Ara-A (as 3% eye ointment) in the topical treatment of herpetic keratitis; and Ara-A (as an intravenous infusion at 15 mg/kg/day for 10 days) in the systemic treatment of herpetic encephalitis [1, 2]. The latter regimen may also be efficacious in the systemic therapy of herpes zoster in the immunosuppressed [3] and of disseminated herpes simplex in the neonate [4]. In the treatment of herpetic keratitis Ara-A has certain advantages over IDU: it is less likely to interfere with corneal wound healing and can be used in patients who have become allergic to IDU or whose infections have become refractory to IDU or Ara-A in the topical therapy of herpetic keratitis and, since it readily penetrates the deeper ocular tissues, it may offer benefit in the topical treatment of deep stromal keratitis and herpetic uveitis.

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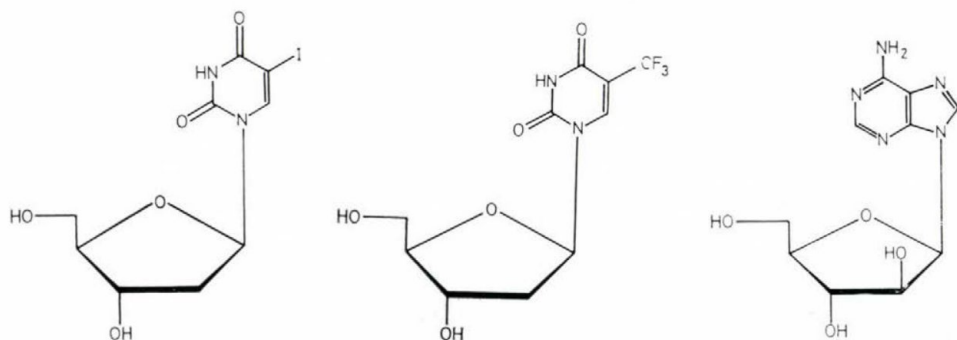
Table I*Nucleoside analogues with antiviral activity**First-generation nucleoside analogues*

IDU	5-iodo-2'-deoxyuridine
TFT	5-trifluoromethyl-2'-deoxyuridine
Ara-A	9- β -D-arabinofuranosyladenine

Second-generation nucleoside analogues

Ara-T	1- β -D-arabinofuranosylthymine
EDU	5-ethyl-2'-deoxyuridine
PDU	5-propyl-2'-deoxyuridine
AIDDU	5'-amino-5-iodo-2',5'-dideoxyuridine
ACG	9-(2-hydroxyethoxymethyl)guanine
BVDU	(E)-5-(2-bromovinyl)-2'-deoxyuridine
FIAC	1-(2-fluoro-2-deoxy- β -D-arabinofuranosyl)-5-iodocytosine
Ribavirin	1- β -D-ribofuranosyl-1,2,4-triazole-3-carboxamide
(S)-DHPA	(S)-9-(2,3-dihydroxypropyl)adenine

At present, herpes simplex encephalitis is the major indication for parenteral Ara-A. To ensure an optimum effect, the drug should be given as early as possible during the course of infection (before the advent of coma) and treatment should only be initiated after the diagnosis has been confirmed by brain biopsy. In a placebo-controlled study of 28 biopsy-proven cases of herpes simplex encephalitis, Ara-A was found to reduce mortality from 70 to 28% [5]. This study has now been extended to over 100 patients (as mentioned in reference [2]), and, although mortality figures have remained low, it cannot be dismissed [5] that more than half of the survivors show moderate to severe neurologic sequelae. Systemic administration of Ara-A is further hampered by its low solubility (0.45 mg/ml at 25 °C), a drawback that necessitates the infusion of very large volumes (> 2 litres/day for a person of 70 kg), with the concomitant risk of fluid overload.

*Fig. 1a. IDU: 5-iodo-2'-deoxyuridine**Fig. 1b. TFT: 5-trifluoromethyl-2'-deoxyuridine**Fig. 1c. Ara-A: 9- β -D-arabinofuranosyladenine*

IDU, TFT and Ara-A are far from specific in their antiviral action. They are all incorporated into DNA of both virus-infected and uninfected cells, cause chromosomal aberrations, suppress bone-marrow functions and produce fetal malformations in experimental animals. IDU and TFT may also pose problems of immunosuppression, mutagenicity, and, possibly, even carcinogenicity. It is obvious that the use of such agents should be restricted to topical therapy, but even so there may be some hazards involved, since topical application of IDU or TFT to the eye may lead to contact dermatitis, follicular conjunctivitis and punctate epithelial keratopathy, and if these toxicities are ignored and treatment continued, then frank epithelial oedema with stromal swelling may arise [6]. Furthermore, IDU has proven to be teratogenic to rabbits when administered topically to the eye in doses similar to those used clinically [7].

It would appear therefore that there is a need for antiviral agents with considerably less toxicity. As succinctly pointed out by PRUSOFF and WARD [6], the ideal antiviral drug should be easy (cheap) to prepare, readily soluble in aqueous medium, chemically stable, metabolically stable, sufficiently apolar, not immunosuppressive, not teratogenic, not mutagenic, not carcinogenic, and, after all, still effective as an antiviral agent. Although one may expect very few, if any, compound to meet all these requirements, some nucleoside analogues of the second-generation (Table I) appear to fulfil at least partially the stringent premises of an ideal antiviral drug. Many of these second-generation compounds take advantage of the fact that some viruses, i.e. herpes simplex virus (HSV) and varicella zoster virus (VZV), induce their own enzymes after they have invaded the cell. Quite often, these virus-induced enzymes, i.e. deoxythymidine (dThd) kinase and DNA polymerase, differ from their cellular counterparts in a number of properties (i.e. substrate specificity), and these differences may offer the molecular basis for the selective antiviral action of the nucleoside analogue. For example, the nucleoside analogue may only be recognized as substrate by the virus-induced dThd kinase, and, as it is only phosphorylated in the virus infected cell, its antiviral action will be restricted to this cell. The virus induced enzyme may not only serve as an activator, as is the case with dThd kinase; it may also act as the target enzyme, as is the case with DNA polymerase. Once it has been converted to its active metabolite, the nucleoside analogue may indeed prove more inhibitory to the viral DNA polymerase than to its cellular counterparts. Thus, virus-encoded enzymes may significantly contribute to the selectivity of nucleoside analogues as antiviral agents, irrespective of the role they serve (activator or target).

How close do the second-generation nucleoside analogues come to achieving the objectives of the ideal antiviral agent? Which viruses are included in their activity spectrum? Are they effective in animal experiments, and, if so, are they also efficacious in humans? Finally, how do these compounds act at

the molecular level? How can their selective antiviral activity be rationalized? For each of the compounds listed we will now attempt to answer these questions. The compounds will first be reviewed one by one, and then we will compare their merits and potentials in a more integrated fashion.

Analytical review of the second-generation drugs

1- β -D-arabinofuranosylthymine (Ara-T): Fig. 2, Table IIa, b;
 5-ethyl-2'-deoxyuridine (EDU), 5-propyl-2'-deoxyuridine (PDU): Fig. 3, Table IIIa, b;
 5'-amino-5-iodo-2',5'-dideoxyuridine (AIDDU): Fig. 4, Table IVa, b;
 9-(2-hydroxyethoxymethyl)guanine (ACG): Fig. 5, Table Va, b;
 (E)-5-(2-bromovinyl)-2'-deoxyuridine (BVDU): Fig. 6, Table VIa, b;
 1-(2-fluoro-2-deoxy- β -D-arabinofuranosyl)-5-iodocytosine (FIAC): Fig. 7, Table VIIa, b;
 1- β -ribofuranosyl-1,2,4-triazole-3-carboxamide (Ribavirin): Fig. 8, Table VIIIa, b;
 (S)-9-(2,3-dihydroxypropyl)adenine [(S)-DHPA]: Fig. 9, Table IXa, b.

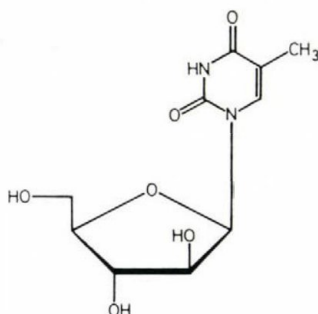


Fig. 2. Ara-T: 1- β -D-arabinofuranosylthymine

Table IIa

Ara-T: Activity spectrum

<i>Cell culture</i>	HSV-1	[8-17]
	HSV-2	[10, 11, 13-15, 17]
	VZV	[12, 18]
	Vaccinia	[8, 14, 17]
	EHV-1	[11, 19]
	FRV	[20]
<i>Animals</i>	HSV-1 keratitis in rabbits	[9]
	EHV-1 hepatitis in hamsters	[11]
	HSV-1 encephalitis in mice	[21]
<i>Humans</i>	—	

Table IIb*Ara-T: Mode of action*

1. Ara-T would be specifically phosphorylated by the virus (HSV, VZV, . . .) encoded dThd kinase, although it may also be phosphorylated in uninfected cells [10–12, 19]
[22, 23]
2. Once phosphorylated to the 5'-triphosphate (ara-TTP) via the 5'-monophosphate (ara-TMP) and the 5'-diphosphate (ara-TDP), it inhibits the DNA polymerase by competition with dTTP [24, 25]
3. Ara-T may be incorporated into cellular DNA (but not into viral DNA) [22]

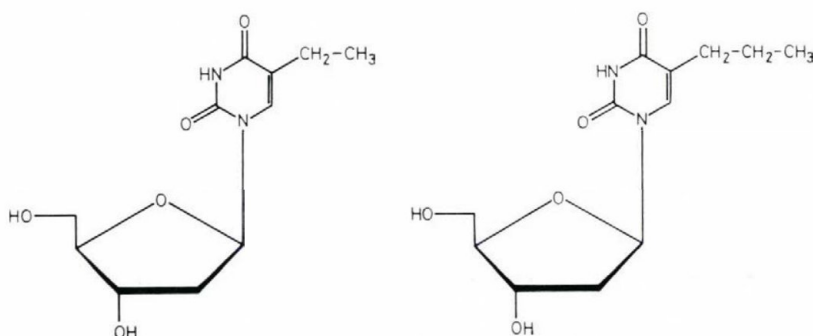


Fig. 3. EDU: 5-ethyl-2'-deoxyuridine; PDU: 5-propyl-2'-deoxyuridine

Table IIIa*EDU, PDU: Activity spectrum*

<i>Cell culture</i>	HSV-1	[17, 26–29]
	HSV-2	[17, 27–29]
	VZV	[18]
	Vaccinia (EDU, not PDU)	[17, 26, 27]
<i>Animals</i>	HSV-1 keratitis in rabbits (EDU)	[30, 31]
	HSV-1 encephalitis in mice (EDU)	[32]
<i>Humans</i>	Herpetic keratitis (EDU)	[33]
	? Herpes labialis	[34]
	? Herpes genitalis	[34]

Table IIIb*EDU, PDU: Mode of action*

1. EDU and PDU are specifically phosphorylated by the virus (HSV, VZV, . . .) encoded dThd kinase [28, 29, 35, 36]
2. PDU inhibits the induction of virus (HSV-1) specific DNase and DNA polymerase, [29]
3. and, although both EDU and PDU may be incorporated into cellular and/or viral DNA, this incorporation would not explain their antiviral action [29, 37, 38]

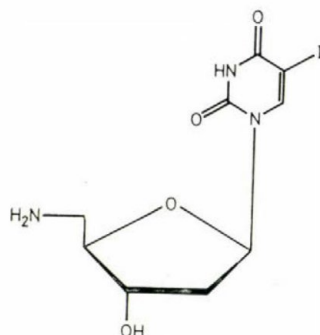


Fig. 4. AIDDU: 5'-amino-5-iodo-2',5'-dideoxyuridine

Table IVa

AIDDU: Activity spectrum

Cell culture	HSV-1	[17, 39]
	HSV-2	[17, 39]
	VZV	[18, 40]
Animals	HSV-1 keratitis in rabbits	[41]
Humans	—	

Table IVb

AIDDU: Mode of action

1. AIDDU is specifically phosphorylated by the virus (HSV-1) encoded dThd kinase (to the 5'-diphosphate), [42]
2. and subsequently incorporated into DNA of HSV-1-infected cells (but not into DNA of uninfected cells) [43]

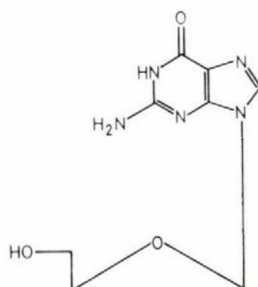


Fig. 5. ACG: 9-(2-hydroxyethoxymethyl)guanine

Table Va*ACG: Activity spectrum*

<i>Cell Culture</i>	HSV-1	[17, 44-46]
	HSV-2	[17, 44-46]
	VZV	[18, 44, 45, 47]
	EBV	[48]
<i>Animals</i>	HSV-1 keratitis in rabbits	[44, 49, 50]
	HSV-1 (and HSV-2) encephalitis in mice	[44, 51, 52]
	HSV-1 skin lesions in guinea pigs	[44, 53]
	HSV-1 ear infection in mice	[54]
	HSV-1 mouth infection in mice	[55]
	HSV-1 ganglion infection in mice	[55, 56]
<i>Humans</i>	Herpetic keratitis	[57-60]
	Localized and disseminated herpes simplex	[61-63]
	Localized and disseminated varicella zoster	[61]

Table Vb*ACG: Mode of action*

1. ACG is specifically phosphorylated by the virus (HSV, VZV, ...) encoded dThd kinase, [64-67]
2. and, once phosphorylated to the triphosphate (ACGTP) via the monophosphate (ACGMP) and the diphosphate (ACGDP), it inhibits the DNA polymerase by competition with dGTP; ACGTP inhibits HSV-1 DNA polymerase to a greater extent than cellular DNA polymerase α [68]
3. ACG may also be incorporated into (viral) DNA, thereby acting as a chain terminator [64, 68, 69]

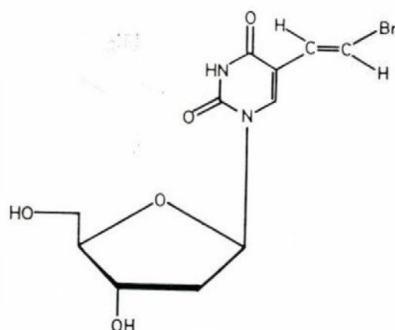
**Fig. 6.** BVDU: (E)-5-(2-bromovinyl)-2'-deoxyuridine

Table VIa*BVDU: Activity spectrum*

<i>Cell culture</i>	HSV-1	[17, 70-75]
	HSV-2	[17, 73-75]
	VZV	[18, 73-75]
	Vaccinia	[17, 70, 74]
	Pseudorabies	[76]
<i>Animals</i>	HSV-1 keratitis in rabbits	[74, 77-79]
	HSV-1 encephalitis in mice	[73, 80]
	HSV-1 skin lesions in guinea pigs	[81]
	HSV-1 skin lesions (and mortality associated therewith) in athymic nude mice	[70, 82]
<i>Humans</i>	Herpetic keratitis	[79]
	Mucocutaneous herpes simplex	[83]
	Localized and disseminated varicella zoster	[84]

Table VIb*BVDU: Mode of action*

1. BVDU would be specifically phosphorylated by the virus (HSV, VZV, . . .) encoded dThd kinase, [74, 75]
2. and, once phosphorylated to the 5'-triphosphate (BVDUTP) *via* the 5'-monophosphate (BVDUMP) and the 5'-diphosphate (BVDUDP), it would inhibit the DNA polymerase by competition with dTTP; BVDUTP would inhibit HSV-1 DNA polymerase to a greater extent than cellular DNA polymerase (α and β) [85]
3. It has not been unequivocally assessed whether BVDU is incorporated into DNA. Unlike IDU, it does not induce the release of oncogenic RNA viruses from mouse carrier cell lines [75, 86]

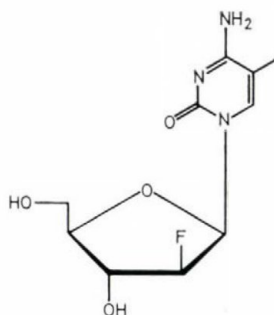
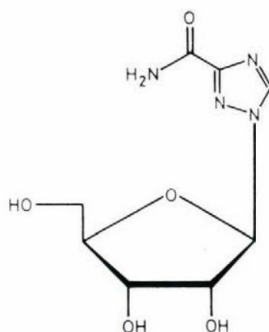
**Fig. 7. FIAC: 1-(2-fluoro-2-deoxy- β -D-arabinofuranosyl)-5-iodocytosine**

Table VIIa*FIAC: Activity spectrum*

<i>Cell culture</i>	HSV-1	[17, 87, 88]
	HSV-2	[17, 88]
	VZV	[18, 88]
	Cytomegalo	[88]
	Vaccinia	[17]
<i>Animals</i>	HSV-1 encephalitis in mice	[88]
<i>Humans</i>	—	

Table VIIb*FIAC: Mode of action*

1. FIAC would be specifically phosphorylated by the virus (HSV, . . .) encoded dThd kinase, [88]
2. and, once phosphorylated to the 5'-triphosphate (FIACTP) *via* the 5-mono-phosphate (FIACMP) and the 5'-diphosphate (FIACDP), it might inhibit the DNA polymerase by competition with dCTP

*Fig. 8.* Ribavirin: 1-β-D-ribofuranosyl-1,2,4-triazole-3-carboxamide**Table VIIIa***Ribavirin: Activity spectrum*

<i>Cell culture</i>	All major RNA and DNA viruses (adeno, herpes, pox, picorna, toga, myxo, rhabdo, retro and reo),	[89-93]
	and, in particular, influenza and parainfluenza viruses	[94-96]
<i>Animals</i>	Influenza and parainfluenza virus infections in mice	[95, 97-99]
	Influenza virus infections in ferrets and squirrel monkeys	[100, 101]
	Lassa fever virus infection in rhesus monkeys	[102]
<i>Humans</i>	Influenza A	[103, 104]

Table VIIIb*Ribavirin: Mode of action*

1. Within the cell ribavirin is readily phosphorylated to its 5'-mono-, 5'-di- and 5'-triphosphate [105]

2. Ribavirin 5'-monophosphate inhibits IMP dehydrogenase. This leads to a depletion of the GTP pool and the inhibition of host cell RNA and DNA synthesis [91, 106-108]
3. Ribavirin 5'-triphosphate selectively inhibits influenza virus RNA polymerase [109, 110]

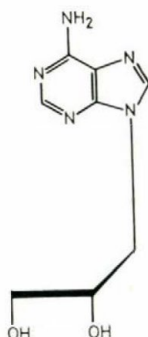


Fig. 9. (S)-DHPA: (S)-9-(2,3-dihydroxypropyl)adenine

Table IXa

(S)-DHPA: Activity spectrum

Cell culture	Several RNA and DNA viruses	[111]
	Vaccinia	[111-113]
	VSV	[111, 112]
	Rotavirus	[93]
	Rous sarcoma virus	[114]
Animals	VSV encephalitis in mice	[111]
	Rabies virus infection in mice	[115]
	Rotavirus infection in mice	[93]
Humans	—	

Table IXb

(S)-DHPA: Mode of action

1. (S)-DHPA inhibits the activity of SAH (S-adenosyl-L-homocysteine) hydrolase, a regulatory enzyme in transmethylation reactions [116]
2. (S)-DHPA inhibits the activity of p60^{src}, a virus-specified protein kinase of 60,000 daltons that is responsible for the transformation of chick embryo fibroblasts by Rous sarcoma virus [114]
3. (S)-DHPA, itself not a substrate for adenosine (or adenine) aminohydrolase, inhibits the deamination of Ara-A and adenosine by adenosine aminohydrolase

Synoptic review of the potentials of nucleoside analogues

Cell culture studies. For most nucleoside analogues (i.e. Ara-T, AIDDU, ACG, BVDU, FIAC) the activity spectrum is virtually restricted to herpesviruses (HSV-1, HSV-2, VZV), and, while some of these nucleoside analogues, viz. Ara-T, BVDU and FIAC, were also found to inhibit viruses other than herpes, for example vaccina, they only did so at concentrations far in excess of those required to inhibit herpesviruses. When tested in primary rabbit kidney cells infected with HSV-1, BVDU surpassed all other nucleoside analogues in potency: thus, in order of decreasing activity, BVDU > FIAC > ACG > IDU > Ara-T > EDU > PDU > TFT > Ara-A > AIDDU [17, 75]. The ID₅₀ (inhibitory dose 50) of BVDU for HSV-1 was ~ 0.01 µg/ml; for AIDDU it was 20–40 µg/ml.

Whereas most nucleoside analogues inhibited HSV-1 and HSV-2 replications to a similar extent, BVDU proved markedly less inhibitory to HSV-2 than to HSV-1: thus, for HSV-2 (in primary rabbit kidney cells), the order of decreasing activity was ACG > FIAC > IDU = EDU > Ara-T > TFT > BVDU > PDU > Ara-A > AIDDU [17, 75]. Since BVDU inhibited HSV-2 replication at a concentration (1 µg/ml) which was at least 100-fold greater than the concentration required to inhibit HSV-1 replication, the compound could serve as a useful tool to discriminate between type 1 and type 2 in the laboratory diagnosis of human herpes simplex virus infections.

The relative activities of the compounds obtained with VZV as the challenge virus (assayed in human diploid fibroblasts) corresponded closely to those noted for HSV-1, thus, in order of decreasing activity: BVDU > Ara-T > ACG = IDU > PDU = TFT > Ara-A > EDU > AIDDU (FIAC was not included in this study) [18, 75]. The ID₅₀ of BVDU for VZV was ~ 0.01 µg/ml; for AIDDU it was 40 µg/ml.

The activity spectrum of ribavirin and (S)-DHPA is entirely different from the activity spectrum of the aforementioned compounds. Ribavirin excels by its activity against influenza [94–96], and arenaviruses (i. e. lassa) whereas (S)-DHPA demonstrated its greatest activity against rhabdoviruses [111].

Animal experiments. In addition to the established antiviral drugs IDU, TFT and Ara-A, several other nucleoside analogues, viz. Ara-T [9], EDU [30, 31], AIDDU [41], ACG [49, 50] and BVDU [77] have proven efficacious in the topical treatment of herpetic keratitis in rabbits. Since these compounds have not been compared under the same experimental conditions, it would be difficult to single out the most effective one. Yet, from their activity relative to IDU, ACG and BVDU would seem to offer the greatest promise for the local therapy of herpetic keratitis.

Several nucleoside analogues, viz. Ara-T [21], EDU [32], ACG [44, 51,

52], BVDU [73, 80] and FIAC [88] have also been found effective in the systemic treatment of herpetic encephalitis in mice, but, it is at present unclear which of these drugs would be the ideal candidate for the therapy of herpetic encephalitis.

In the topical treatment of herpetic skin lesions in athymic nude mice, ACG and BVDU proved equally effective, and significantly more effective than their congeners Ara-T, EDU and AIDDU [82].

In agreement with the cell culture findings, ribavirin has proven efficacious in the treatment of influenza (and parainfluenza) virus infections in mice, ferrets and monkeys [95, 97–101], whereas (S)-DHPA conferred some protection against rabies [115]. Quite intriguing is the protective effect produced by ribavirin in rhesus monkeys infected with Lassa fever virus [102].

Human trials. From the cell culture and animal experiments ACG and BVDU have emerged as the most promising anti-herpes agents and preliminary clinical trials indicate that these compounds may also be effective in various herpesvirus manifestations in humans: i.e. ACG (3% eye ointment) [57–60] and BVDU (0.1% eye drops) [79] in the topical treatment of herpetic keratitis; ACG (5% ointment) [63] and BVDU (1% ointment) [83] in the topical treatment of mucocutaneous herpes lesions; ACG (15 mg/kg/day, intravenously) [61] and BVDU (7.5 mg/kg/day, orally) [84] in the systemic treatment of localized and disseminated herpes simplex and varicella zoster virus infections in cancer patients. Since both ACG [44] and BVDU [119] are readily absorbed from the gut, oral administration may well be the ideal route in the systemic therapy of HSV and VZV infections (Table X).

Ribavirin given orally to human volunteers at 1 g/day had no effect on preventing mild, common cold-type symptoms of influenza A infection, but did have an effect on fever and symptoms of moderate-to-severe illness paralleled by a diminution in the quantity of virus shed [103]. The efficacy of ribavirin might further be improved if administered as an aerosol by the intranasal route. This route of administration would maximize drug levels in the respiratory tract mucosa while minimizing systemic toxicity.

Mode of action. Most of the second-generation drugs are selective anti-herpes compounds and they display remarkable similarities in their mode of action (Fig. 10). First, they are specifically phosphorylated by the virus-encoded dThd kinase: Ara-T [10–12, 19], EDU, PDU [28, 29, 35, 36], AIDDU [42], ACG [64–67], BVDU [74, 75] and FIAC [88]. The resulting product may be the 5'-monophosphate, or the 5'-diphosphate [42], since the virus-encoded dThd kinase may also be endowed with dTMP kinase activity [120, 121]. The 5'-mono or 5'-diphosphates would then be converted by cellular kinases to the 5'-triphosphates. Ara-A is also converted to the 5'-triphosphate (Ara-ATP) but this phosphorylation process entirely depends on cellular kinases. Second, once they have been converted to their 5'-triphosphate form,

Table X

*Major indications for clinical use of nucleoside analogues**First-generation drugs (approved for clinical use)*

IDU (0.1% eye drops): herpetic keratitis

TFT (1% eye drops): herpetic keratitis

Ara-A (3% eye ointment): herpetic keratitis

Ara-A (intravenous infusion, 15 mg/kg/day for 10 days): herpetic encephalitis

Second-generation drugs (not yet approved for clinical use)

Ara-T: not defined yet

EDU, PDU (topical): localized HSV lesions (?)

ACG (topical and systemic): localized and generalized HSV and VZV infections

BVDU (topical and systemic): localized and generalized HSV and VZV infection

FIAC: not yet defined

Ribavirin (topical and systemic): influenza, lassa and other arenavirus infections (?)

Ara-A [122], ACG [68], BVDU [85] and, possibly, FIAC may specifically inhibit the viral DNA polymerase, Ara-ATP in competition with dATP, ACGTP in competition with dGTP, BVDUTP in competition with dTTP, and FIACTP in competition with dCTP (Fig. 10). Ara-TTP would also interfere with DNA polymerase activity, but does apparently not discriminate between viral and cellular DNA polymerases [24, 25]. AIDDUTP would not interfere with DNA polymerase activity; it is directly incorporated into DNA (of the virus-infected cell) [43]. For EDU and PDU the target (enzyme) has not yet been elucidated. One may postulate, therefore, that compounds like ACG and BVDU owe their selective anti-herpes activity to at least two viral enzymes, dThd kinase and DNA polymerase. For Ara-T and AIDDU the selectivity would only reside in the viral dThd kinase, while the selectivity of Ara-A would depend only on the viral DNA polymerase.

Several nucleoside analogues, *viz.* IDU [6, 123], TFT [6, 124], Ara-A [125], Ara-T [22], EDU, PDU [29, 37, 38], AIDDU [43] and ACG [64, 68, 69],

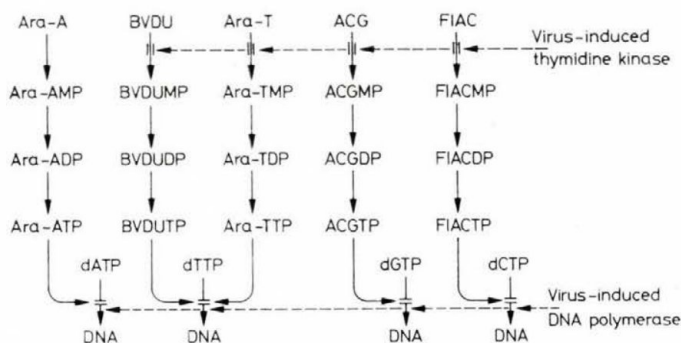


Fig. 10. Mechanism of inhibitory activity of principal anti-herpes compounds on the replication of HSV and VZV

can be incorporated into DNA, be it cellular or viral DNA. For some analogues [i.e. IDU and AIDDU], this incorporation may provide a plausible basis for their antiviral action. Indeed, there is a strong parallelism between antiviral activity and the degree of incorporation of IDU and AIDDU into HSV-1 DNA [126]. The incorporation of AIDDU into HSV-1 DNA leads to both single-stranded and double-stranded breaks due to the presence of the extremely labile phosphoramidate bonds. The role of this DNA damage in the antiviral activity of AIDDU is unclear since inhibition of HSV replication by IDU is achieved in the absence of such strand breakage [126].

ACG is incorporated into DNA of HSV-transformed cells [69] and, since ACG lacks a 3'-hydroxyl group, this incorporation leads to chain termination and the accumulation of short nascent DNA chains. Thus, the incorporation of ACG into DNA may at least partially account for its cytotoxic effects on HSV-transformed cells.

For other compounds, i.e. Ara-A, Ara-T, EDU and PDU, the incorporation into DNA may not necessarily affect the functioning of the DNA. For these compounds the relationship between antiviral activity and incorporation into DNA would seem rather fortuitous.

The mechanism of action of ribavirin and (S)-DPHA is fundamentally different from that of the other compounds. In its 5'-monophosphate form ribavirin inhibits IMP dehydrogenase, a key enzyme in purine mononucleotide biosynthesis. This inhibitory effect may not only explain the broad-spectrum antiviral activity of the drug but also its cytotoxicity [127], its teratogenicity [128] and its immunosuppressive [129] and antitumour [130] properties. The more selective nature of the inhibitory activity of ribavirin on influenza virus infections may be related to the drug's capacity to specifically inhibit influenza RNA polymerase [109, 110]. For (S)-DPHA, the precise mechanism of action remains to be elucidated. The compound interferes at several metabolic reactions [114, 116-118]. Its principal action may well be the inhibition of SAH (S-adenosyl-L-homocysteine) hydrolase [116]. SAH is an effective inhibitor of SAM (S-adenosylmethionine)-dependent methylation reactions. When SAH hydrolase is blocked, SAH would accumulate. This may, in turn, lead to an inhibition of the methylation reactions, and, as far as these transmethylation reactions are required for the maturation of viral mRNA, the inhibition of SAH hydrolase may finally be revealed by an inhibition of the viral replication cycle.

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DIFFERENTIAL INHIBITION OF HERPES SIMPLEX VIRUSES, TYPE 1 (HSV-1) AND TYPE 2 (HSV-2), BY (*E*)-5-(2-X-VINYL)-2'-DEOXYURIDINES*

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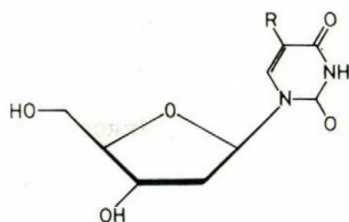
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In recent years, several new compounds have been developed which exhibit a potent and selective anti-herpes activity. These compounds include 9-(2-hydroxyethoxymethyl)guanine (acycloguanosine) [1, 2], 1-(2-fluoro-2'-deoxy- β -D-arabinofuranosyl)-5-iodocytosine (2'-fluoro-5-iodoaracytosine) [3] and (*E*)-5-(2-bromovinyl)-2'-deoxyuridine [(*E*)-5-(2-bromovinyl)-dUrd] [4]. At least two factors appear to contribute to the specificity of acycloguanosine as an inhibitor of herpes simplex virus type 1 (HSV-1); (i) the virus-specific deoxythymidine (dTd) kinase which recognizes acycloguanosine as a substrate, thereby confining the action of the compound to the virus-infected cell [5]; and (ii) the virus-induced DNA polymerase which is more efficiently inhibited by the triphosphate of acycloguanosine than the cellular DNA polymerase α [6]. Preliminary evidence suggests that (*E*)-5-(2-bromovinyl)-dUrd, like acycloguanosine, is specifically phosphorylated in the HSV-infected cell, and, once converted to its 5'-triphosphate, it inhibits viral DNA polymerase more effectively than cellular DNA polymerase α and β [7].

(*E*)-5-(2-bromovinyl)-dUrd and its two 5-(2-halogenovinyl) analogues, (*E*)-5-(2-iodovinyl)-dUrd and (*E*)-5-(2-chlorovinyl)-dUrd have also been found to inhibit herpes simplex virus type 2 (HSV-2), albeit at a 100 to 200-fold higher concentration than that required to inhibit HSV-1 replication [8, 9]. These observations have now been extended to several other 5-substituted 2'-deoxyuridines including (*E*)-5-(1-propenyl)-dUrd, (*E*)-5-(3,3,3-trifluoro-1-propenyl)-dUrd and (*E*)-5-(2-cyanovinyl)-dUrd. All these compounds were evaluated for their inhibitory effect on HSV-1 and HSV-2 replication. In these tests, 5-iodo-, 5-ethyl-, 5-propyl- and 5-trifluoromethyl-dUrd served as reference materials.

The structural formulae of these compounds are seen in Fig. 1. The only difference between (*E*)-5-(2-iodovinyl)-dUrd and 5-iodo-dUrd, and between (*E*)-5-(3,3,3-trifluoro-1-propenyl)-dUrd and 5-trifluoro-methyl-dUrd is the

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R = -I	:	5-iodo-dUrd
-CH ₂ -CH ₃	:	5-ethyl-dUrd
-CH ₂ -CH ₂ -CH ₃	:	5-propyl-dUrd
-CF ₃	:	5-trifluoromethyl-dUrd
	:	(E)-5-(2-bromovinyl)-dUrd
	:	(E)-5-(2-iodovinyl)-dUrd
	:	(E)-5-(2-chlorovinyl)-dUrd
	:	(E)-5-(1-propenyl)-dUrd
	:	(E)-5-(3,3,3-trifluoro-1-propenyl)-dUrd
	:	(E)-5-(2-cyanovinyl)-dUrd

Fig. 1. Structural formulae of 5-substituted 2'-deoxyuridines

presence (or absence) of an ethene bridge that links the iodine or trifluoromethyl group to the uracil ring. This ethene bridge is also present in (*E*)-5-(2-bromovinyl)-dUrd, (*E*)-5-(2-chlorovinyl)-dUrd, (*E*)-5-(1-propenyl)-dUrd and (*E*)-5-(2-cyanovinyl)-dUrd, but not in 5-ethyl-dUrd or 5-propyl-dUrd. From our findings it would appear that the presence of the connecting ethene chain has a dramatic impact on anti-herpes activity.

Compounds, viruses and antiviral assays

The origin of the compounds is indicated in reference [8]: 5-iodo-, 5-ethyl-, 5-propyl-, 5-trifluoromethyl-, (*E*)-5-(2-bromovinyl)-, (*E*)-5-(2-iodovinyl)-dUrd; in reference [9]: (*E*)-5-(2-chlorovinyl)-dUrd; and in references [10, 11]: (*E*)-5-(1-propenyl)-dUrd, (*E*)-5-(3,3,3-trifluoro-1-propenyl)-dUrd. The synthesis of (*E*)-5-(2-cyanovinyl)-dUrd will be described elsewhere [12].

The origin of the herpes viruses was as follows: HSV-1 (KOS), HSV-2 (LYONS) and HSV-2 (196) were received from Dr. W. E. RAWLS (Baylor University College of Medicine, Houston, Tex. USA); HSV-1 (F), HSV-1 (McIntyre) and HSV-2 (G) from the American Type Culture Collection (Rockville, Md. USA); VEX 142, VEX 149, VEX 538, VEO 231, VEW 257, VDO 374, VDX 348, VDX 500, VCX 210, VCX 301, VCX 304 and VDX 192 were directly isolated from patients. All HSV stocks were prepared in primary rabbit kidney (PRK) cell cultures. The clinical isolates were characterized as type 1 or 2 according to (i) their origin (oral or genital); (ii) the virus yields attained in PRK cells; (iii) the plaque-forming ability in chick-embryo cells, and (iv) neutralization by type-specific anti-herpesvirus serum [8].

The antiviral assays were performed in confluent PRK cell cultures which had been inoculated with 100 CCID₅₀ (cell culture infecting dose-50) of virus; after a 1 h adsorption period, residual virus was removed and all the cell cultures were incubated with Earle's minimal essential medium containing 3% fetal bovine serum and varying concentrations of the compounds. Antiviral activity was expressed as ID₅₀ (inhibitory dose-50), i.e. the concentration required to reduce viral cytopathogenicity by 50%, as soon as it had attained completion (100% cell destruction) in the control (virus-infected untreated) cell cultures.

Results

(E)-5-(2-bromovinyl)-dUrd, (E)-5-(2-iodovinyl)-dUrd, (E)-5-(2-chlorovinyl)-dUrd, (E)-5-(1-propenyl)-dUrd and (E)-5-(3,3,3-trifluoro-1-propenyl)-dUrd proved to be potent inhibitors of HSV-1 replication: their average ID₅₀ for the three HSV-1 strains KOS, F and McIntyre were 0.008, 0.008, 0.018, 0.08 and 0.08 µg/ml, respectively; these values compared favourably with the ID₅₀ values obtained for the reference compounds 5-iodo-dUrd, 5-ethyl-dUrd, 5-propyl-dUrd and 5-trifluoromethyl-dUrd (Table I). Only (E)-5-(2-cyanovinyl)-dUrd inhibited HSV-1 replication at an ID₅₀ that was significantly higher than the ID₅₀ at which the other compounds inhibited HSV-1 replication.

5-Iodo-dUrd, 5-ethyl-dUrd, 5-propyl-dUrd and 5-trifluoromethyl-dUrd inhibited HSV-2 at a concentration which did not markedly vary from the concentrations that were required to inhibit HSV-1 replication. However, for

Table I

Antiviral activities of (E)-5-(2-X-vinyl)-dUrd analogues in primary rabbit kidney cell cultures, as assessed with 3 laboratory strains of HSV-1 and 3 laboratory strains of HSV-2

Compound	ID ₅₀ (µg/ml)*					
	HSV-1			HSV-2		
	KOS	McIntyre	F	Lyons	G	196
5-Iodo-dUrd	0.15	0.15	0.15	0.2	0.4	0.2
5-Ethyl-dUrd	0.8	0.7	0.4	0.5	0.4	0.2
5-Propyl-dUrd	0.9	0.5	0.2	2	3	3
5-Trifluoromethyl-dUrd	1	0.7	0.7	0.7	0.3	0.6
(E)-5-(2-Bromovinyl)-dUrd	0.007	0.009	0.007	1	1	1.5
(E)-5-(2-Iodovinyl)-dUrd	0.01	0.01	0.004	1	1	4
(E)-5-(2-Chlorovinyl)-dUrd	0.02	0.015	0.02	4	2	1.5
(E)-5-(1-Propenyl)-dUrd	0.06	0.15	0.03	8	11	27
(E)-5-(3,3,3-Trifluoro-1-propenyl)-dUrd	0.06	0.13	0.05	33	50	80
(E)-5-(2-Cyanovinyl)-dUrd	4	4	4	200	200	200

* ID₅₀ = 50% inhibitory dose or concentration required to inhibit virus-induced cytopathogenicity by 50%. For 5-iodo-dUrd, 5-ethyl-dUrd, 5-propyl-dUrd, 5-trifluoromethyl-dUrd, (E)-5-(2-bromovinyl)-dUrd and (E)-5-(2-iodovinyl)-dUrd, the data were taken from refere

Table II

Differential susceptibility of HSV-1 and HSV-2 to the inhibitory effects of (E)-5-(2-X-vinyl)-dUrd analogues in primary rabbit kidney cell cultures

Compound	Ratio $\left(\frac{\text{ID}_{50} \text{ for HSV-2}}{\text{ID}_{50} \text{ for HSV-1}} \right)^*$
5-Iodo-dUrd	1.8
5-Ethyl-dUrd	0.6
5-Propyl-dUrd	5
5-Trifluoromethyl-dUrd	0.7
(E)-5-(2-Bromovinyl)-dUrd	152
(E)-5-(2-Iodovinyl)-dUrd	250
(E)-5-(2-Chlorovinyl)-dUrd	136
(E)-5-(1-Propenyl)-dUrd	192
(E)-5-(3,3,3-Trifluoro-1-propenyl)-dUrd	679
(E)-5-(2-Cyanovinyl)-dUrd	55

* Calculated from ID_{50} values for 3 laboratory strains of HSV-1 and 3 laboratory strains of HSV-2 (see Table I)

Table III

Susceptibility of different clinical isolates of HSV-1 and HSV-2 to the inhibitory effects of (E)-5-(3,3,3-trifluoro-1-propenyl)-dUrd in primary rabbit kidney cell cultures

Virus isolate	ID_{50} ($\mu\text{g/ml}$)*
<i>HSV-1</i>	
VEX 142	0.04
VEX 149	0.07
VEX 538	0.07
VEO 231	0.04
VEW 257	0.04
VDO 374	0.04
VDX 348	0.07
VDX 500	0.04
Average for all HSV-1 isolates	0.05
<i>HSV-2</i>	
VCX 210	40
VCX 301	40
VCX 304	70
VDX 192	40
Average for all HSV-2 isolates	47
Ratio $\left(\frac{\text{ID}_{50} \text{ for HSV-2}}{\text{ID}_{50} \text{ for HSV-1}} \right)$	940

* ID_{50} = 50% inhibitory dose or concentration required to inhibit virus-induced cytopathogenicity by 50%

(E)-5-(2-bromovinyl)-dUrd, (E)-5-(2-iodovinyl)-dUrd, (E)-5-(2-chlorovinyl)-dUrd, (E)-5-(1-propenyl)-dUrd, (E)-5-(3,3,3-trifluoro-1-propenyl)-dUrd and (E)-5-(2-cyanovinyl)-dUrd, the concentrations required to inhibit HSV-2 replication were significantly higher than those required for HSV-1 inhibition (Table I). For (E)-5-(2-bromovinyl)-dUrd, the ID_{50} for HSV-2 replication was about 150 times higher than the ID_{50} for HSV-1 replication (Table II); for (E)-5-(1-propenyl)-dUrd this ratio amounted to 192, for (E)-5-(2-iodovinyl)-dUrd it was 250, and for (E)-5-(3,3,3-trifluoro-1-propenyl)-dUrd it reached 679.

In addition to the laboratory strains, clinical isolates of HSV-1 and HSV-2 showed also remarkable differences in susceptibility towards inhibition by (E)-5-(2-bromovinyl)-dUrd, (E)-5-(2-iodovinyl)-dUrd, (E)-5-(2-chlorovinyl)-dUrd, (E)-5-(1-propenyl)-dUrd, (E)-5-(3,3,3-trifluoro-1-propenyl)-dUrd and (E)-5-(2-cyanovinyl)-dUrd. Again, the greatest divergence was obtained with (E)-5-(3,3,3-trifluoro-1-propenyl)-dUrd, which inhibited HSV-1 isolates at a concentration almost 1000 times lower than the concentration at which HSV-2 isolates were inhibited (Table III).

Discussion

The differential sensitivity of HSV-1 and HSV-2 to (E)-5-(2-halogenovinyl)-dUrd, (E)-5-(2-cyanovinyl)-dUrd, (E)-5-(1-propenyl)-dUrd and, in particular, (E)-5-(3,3,3-trifluoro-1-propenyl)-dUrd, could be advocated as a useful marker test for the rapid and specific diagnosis of human HSV-1 and HSV-2 infections. Such marker tests would offer the advantage of being less time-, labour- and reagent-consuming than many of the previously described typing techniques including restriction endonuclease mapping [13, 14].

The precise mechanism by which (E)-5-(2-halogenovinyl)-dUrd, (E)-5-(2-cyanovinyl)-dUrd, (E)-5-(1-propenyl)-dUrd and (E)-5-(3,3,3-trifluoro-1-propenyl)-dUrd exert their anti-HSV activity and the reason(s) for their discriminating behaviour towards HSV-1 and HSV-2 remain to be explored. Obviously, several factors may contribute to the specificity of these compounds as HSV-1 inhibitors, for example the virus-encoded dThd kinase (as activating enzyme) and the viral DNA polymerase (as target enzyme). For (E)-5-(2-bromovinyl)-dUrd the kinetic constants as inhibitor of dThd kinase have recently been estimated with both HSV-1 and HSV-2-encoded dThd kinase as the enzyme source: the K_i values amounted to 0.24 and 4.24 μM , respectively [15]. Thus, the differences in susceptibility of HSV-1 and HSV-2 replication to inhibition by (E)-5-(2-bromovinyl)-dUrd could at least partially be accounted for by differences in substrate affinity of 5-(2-bromovinyl)-dUrd for the virus-induced dThd kinase.

That (E)-5-(3,3,3-trifluoro-1-propenyl)-dUrd, (E)-5-(1-propenyl)-dUrd and (E)-5-(2-cyanovinyl)-dUrd resemble the (E)-5-(2-halogenovinyl)-dUrd

analogues in their discriminating behaviour towards HSV-1 and HSV-2 is not surprising if one considers the structural similarity between these compounds (Fig. 1): at C-5 of the uracil ring they all carry a vinyl group of which the hydrogen in the E ("Entgegen") position of C-2 is replaced by an halogen, a cyano, a methyl or a trifluoromethyl group. Thus, they can all be regarded as (*E*)-5-(2-X-vinyl)-dUrd analogues, and the (*E*)-(2-X-vinyl) substituent may well be a critical determinant in the discriminating behaviour of these compounds towards HSV-1 and HSV-2. It is possible that other *E*-5-(2-X-vinyl)-dUrd analogues, e.g. with X = F, NO₂, etc., would be endowed with similar properties.

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ANTIVIRAL AGENTS: THE ROAD FROM SCEPTICISM TO EFFICACY*

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When the history of antiviral agents is written, the decade of the 60s will be remembered as a period of hope and expectation mixed with considerable scepticism; the 70s as the beginning of demonstrated antiviral efficacy and a change from scepticism to guarded optimism; the 80s, it is hoped, will be the exciting period of development of new drugs with proven efficacy against several classes of viruses.

After a slow start, amantadine is now widely accepted for prophylaxis and has demonstrated potential for therapy against influenza; adenine arabinoside is licensed for the treatment of herpes encephalitis and its potential in other areas has been demonstrated; adenine arabinoside monophosphate may be a clinically more convenient form of the drug; acyclovir is undergoing clinical testing following very promising preliminary studies; ribavirin may prove of value in the treatment of arenavirus infections and interferon has entered a phenomenal phase of interest and worldwide publicity. Because of the positive studies in infectious diseases (influenza, rhinovirus, zoster, chronic viral hepatitis), and preliminary reports of benefit in cancer, interferon has received wide publicity in the lay press. The danger is over promise. Although interferon investigators have held high hope for its clinical application, none believe it to be the penicillin of viral disease. There should be little doubt that it will have a role in clinical medicine, most probably in combination with other antivirals, cancer drugs, and supportive treatment.

Interferon

Interferon (IFN) received its initial acclaim as a potential potent, broad spectrum antiviral. A major factor contributing to clinical studies in the U.S. has been the availability of exogenous leukocyte interferon produced through the efforts of Dr. KARI CANTELL and his associates at the Finnish Red Cross Blood Transfusion Service. For many years this was the only source of interfe-

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ron for clinical use, but with the recent interest in cancer therapy, a number of potential producers have been developed in the U.S. The early studies of Dr. THOMAS MERIGAN in zoster and chronic hepatitis B have contributed significantly to knowledge of the pharmacokinetics of interferon [1]. This, together with preliminary studies in cancer patients by HANS STRANDER and others, has led to considerable interest in testing interferon against various forms of cancer. Such interest, in turn, led to purchase by the American Cancer Society of considerable amounts of interferon to support such studies. The past fiscal year has also seen the establishment of a Biological Response Modifier Program in the National Cancer Institute to support similar studies.

Most of the studies performed and planned in the U.S. have been done with α interferon (leukocyte), although the NCI is in the process of obtaining lymphoblastoid and β interferon (fibroblast) as well. It is clear that these interferons behave differently. For example, experiments at the University of Leuven [2] have shown that only when β interferon is administered by the intravenous route can levels comparable to α interferon administered intramuscularly be attained. Therefore, it is important to do comparative studies and not to assume that results using one type can be applied to the other. It should also be noted that all studies in the U.S. are currently being performed with at least 10^6 units IFN/mg protein.

Infectious disease studies have focussed on treatment of herpes, hepatitis B and viral respiratory infections. It is estimated that about 150 patients have taken part in herpes zoster protocols. The efficacy of α interferon against shingles in the immunocompromised patient has been reported [3]. These studies are continuing, examining the effect of interferon against varicella in high risk patients. Other herpes studies under way include those of PAZIN and associates [4] who have examined the prophylactic potential of α interferon in patients with a 60% chance of serious herpes recurrence following microsurgery for trigeminal neuralgia. To date, 37 patients have been followed in a double-blind, placebo-controlled clinical trial. Nineteen patients received interferon at a dose of 7×10^4 units/kg/day starting on the day prior to surgery and continuing for four days after surgery. Eighteen patients were enrolled in the placebo group. All patients undergoing neurovascular surgery received high doses of steroids. The total frequency of reactivation in the placebo group was 83% (15/18) as compared to 47% (9/19) in the interferon group. There was also a highly significant reduction in the frequency and duration of virus shedding.

There have been conflicting reports on the role of interferon in prevention or treatment of upper respiratory tract infections. Some maintain that low doses (approximately 2000 U) are sufficient for prophylaxis, whereas other studies indicate that high dosages are needed (10^6 U). If interferon is to be clinically useful, it should be efficacious when taken at the time of exposure

and not require frequent follow-up therapy. A clinical study is under way to determine whether two doses of interferon (10^6 U) prior to experimental challenge with rhinovirus and two doses post challenge will be sufficient to abort infection. Preliminary evidence indicates that this treatment schedule is insufficient. At the present time, we do not see much promise for interferon as a preventive for the common cold or influenza.

The work with chronic active hepatitis B has been of special interest, not only for the benefit of these specific patients who may transmit the disease to contacts, are in danger of cirrhosis of the liver (and possibly hepatoma), but also as a model for the role of interferon in chronic diseases. MERIGAN and his associates at Stanford have been the leaders in these studies. To date, they have treated 38 patients with chronic active hepatitis B and found that premenopausal women appear to be the best candidates for antiviral treatment. The majority of hepatitis B carriers are, however, men; thus, studies at Stanford are focussed on developing regimens that will be effective in men as well as women. Currently, the best results are obtained using pulses of combined therapy with adenine arabinoside (ara-A) and interferon (at 7.5×10^4 IFN- α U/kg/day and 5–10 mg ara-A/kg/day) followed by a maintenance course with interferon lasting several months. On this regimen, 8 of 16 patients have shown a reduction of Dane particles, a fall in hepatitis B e antigen below the level of detection, a partial reduction of surface antigen, and disappearance of the core antigen from liver biopsy. Although eradication of all signs of carriage are desirable, the type two response described by Merigan wherein there is a permanent eradication of Dane particles and e antigen with only a partial reduction of surface antigen is sufficient to eradicate the infectivity of the sera of these patients for chimpanzees. Prior to initiation of a placebo-controlled trial, studies to evaluate the role of adenine arabinoside monophosphate or acyclovir as substitutes for ara-A will be performed. In addition, the pharmacokinetic interaction of interferon administration associated with prolonged blood levels of ara-A derivatives requires further study. Similar studies by WEIMER and associates [5] noted only a transient drop in DNA polymerase with interferon treatment. One difference between these studies was in the total dosage, the daily dose being several times higher in the studies of MERIGAN *et al.* [6] and given for longer periods of time. Further, MERIGAN *et al.* now feel that for enduring effects on carriage, combined treatment with interferon plus another drug such as adenine arabinoside is important.

One of the biggest problems in organ transplantation is associated viral infection. Investigators at the Massachusetts General Hospital are examining the role of IFN- α against a variety of virus infections in renal transplant patients. Preliminary results [7] indicate that IFN- α could alter cytomegalovirus (CMV) infections in patients who were seropositive prior to transplantation. IFN- α was given to the treatment group at 3×10^6 units twice a week for a total

of 15 doses. IFN- α helped to prevent the occurrence of CMV viraemia and clinical illnesses when the patient was not simultaneously receiving anti-thymocyte globulin. Further studies are under way to determine the optimal dosage schedules of IFN- α for both primary CMV infections and reactivated CMV disease in renal transplant patients. These same investigators have observed a reduction in Epstein-Barr virus excretion in patients treated with interferon.

Investigators at Sloan-Kettering and the Fred Hutchinson Cancer Institute are doing preliminary trials with IFN- α to prevent viral infections after marrow transplantation. These studies are relatively new, and it is too early to present results.

As a direct result of these encouraging studies, together with preliminary reports of benefit in cancer patients by HANS STRANDER and others, a number of clinical trials in patients with selected cancers have been initiated. The American Cancer Society (ACS) has obtained a large quantity of interferon, and four clinical protocols have been designed to treat at least 30 patients per protocol. Melanoma will be studied at UCLA, Sloan-Kettering and Yale; lymphoma at M. D. Anderson, Stanford and Sloan-Kettering; breast cancer at Mt. Siani, Roswell Park and University of Wisconsin, and myeloma at Columbia, Johns Hopkins, and M. D. Anderson. Of particular note are preliminary studies in breast cancer under the guidance of JORDAN GUTTERMAN at M. D. Anderson in Houston, Texas. Eleven patients were treated for eight weeks with 3×10^6 units daily intramuscularly, and six patients received 9×10^6 units daily for eight weeks. Patients achieving remission were maintained on 3×10^6 units three times a week. GUTTERMAN reports that 5/11 patients on low dose and 2/6 on high dose achieved tumor regression. Regression was measured in soft tissue, bone, and bone marrow. Remissions have been sustained for a period ranging from 8 weeks to $60 \pm$ weeks (median — 27 weeks) on maintenance interferon administered three times weekly. One of the seven responders is still in partial remission (GUTTERMAN, personal communication). Based on these promising results, more extensive ACS studies are currently under way. Criteria for inclusion in the study are histologically confirmed metastatic breast cancer with measurable disease in lymph nodes, cutaneous tissue, or lung. Patients are to be treated daily for 28 days with 3×10^6 units of IFN- α ; those achieving a partial response ($\pm 50\%$ reduction of the measured tumour) will be randomized for either continued treatment or observation. Those to be continued will receive 3×10^6 units daily for up to an additional 28 days, then observed until objective prognosis can be determined. These studies will also attempt to compare intramuscular versus subcutaneous routes of administration. If no response is observed in 28 days, the patient will be continued on treatment for an additional 14 days, at which time treatment will be discontinued if no progress is seen.

MERIGAN *et al.* [8] published preliminary observations on the effect of IFN- α in non-Hodgkin's lymphoma. To date they have treated six patients who had received no prior treatment. In four of these, there was a highly significant shrinkage of the tumour masses. In the other patients, there was clearly rapidly advancing disease. They have also treated two other patients with the standard regimen (10^7 U/day for 30 days) who had previously received cytotoxic chemotherapy, specifically cytoxan, vincristine, and prednisone. One of the patients also responded with dramatic regression of his tumour. GUTTERMAN has also observed beneficial effects in 6/11 patients with non-Hodgkin lymphoma. Three of the six have complete or partial remission; three others show some improvement.

The ACS studies are evaluating three dosages in a blind manner: 1, 3, and 10×10^6 U/day intramuscularly for 30 days. It is proposed that nine patients with each of five major histological types of lymphoma be studied, a total of 45 patients, 15 at each dosage level. The patients, with a confirmed histological diagnosis, will not have had prior chemotherapy, radiation therapy, or corticosteroid therapy for 30 days. The measurable disease must have been stable or progressive during the 30 days prior to interferon therapy. These patients will receive no other anti-tumour therapy of any kind during the 30 days of interferon therapy and for 30 days thereafter. If such treatment is warranted, the patient will be considered a no response.

A similar study is in progress, utilizing IFN- α in patients with measurable skin and/or lung metastases of malignant melanoma. Interferon is to be administered intramuscularly daily for 42 days in one of three dosages (1, 3, or 10×10^6 U). As in the lymphoma study, no other anti-tumour treatment will be given during the 42 days nor for the following 30-day observation period; if treatment becomes necessary, the patient will be considered to have experienced progression.

In an open, preliminary study, STRANDER has reported apparent remissions in 4/4 myeloma patients treated with 3×10^6 units daily for 3 to 19 months. GUTTERMAN has also observed beneficial effects in 6/10 myeloma patients treated with $3-9 \times 10^6$ units; 5/10 patients responded with a $\pm 50\%$ decrease in serum myeloma protein and/or Bence-Jones protein. Bence-Jones protein disappeared in one patient. The present ACS study hopes to confirm these preliminary findings in patients with active or progressive "typical" myeloma with overt skeletal lesions, monoclonal gammopathy, marrow plasmocytosis and anaemia with an estimated survival of more than six months. All patients receive an initial dosage of 3×10^6 U/day; if granulocytopenia ($< 2000/\text{mm}^3$) has not developed in 14 days, the dose will be increased to 6×10^6 U/day. Treatment is continued for six months unless the disease is clearly progressing.

After discontinuance of treatment, patients will be followed without therapy until relapse (greater than 20% increase in serum M protein and/or

doubling of Bence-Jones proteinuria). Thus far, 11 patients have been on treatment for 1-1/2 to 6 months; disease regression has been observed in 3 cases. The most significant regression was seen in a patient with the greatest side effects (pancytopenia, haemolytic anaemia and abnormal liver function test). Two cases appeared stable during treatment and relapsed abruptly when interferon was discontinued.

In addition to the clinical studies utilizing exogenous interferon, scientists from the NCI and NIAI are testing the efficacy of the inducer poly (I): poly (C) complexed with poly-L-lysine and carboxymethylcellulose (poly ICLC) in the treatment of a variety of cancers including myeloma and juvenile papilloma (ARTHUR LEVINE, HILTON LEVY, personal communication). This synthetic double-stranded RNA is resistant to ribonucleases in primate sera and is a potent IFN inducer. Poly ICLC can produce interferon serum levels 100-fold greater than those found effective with exogenous interferon. The response, however, is not uniform and the inducer can be quite toxic. It is still too early to evaluate its clinical efficacy and potential.

Expanded interest in IFN has led to increased studies on the biological properties of IFN other than its role as an antiviral agent. As noted, particular emphasis is being placed on the determination of its mechanism of action as an anti-cancer drug. Considerable information is being developed on the immunoregulatory functions of IFN. These include regulatory effects on lymphocytes and macrophages, activation of natural killer cells, cell growth inhibition, tumoricidal activity, and suppression and enhancing of the immune response.

Interferon studies are further complicated by the existence of a third type of interferon, IFN- γ (immune interferon). This differs significantly from IFN- α and IFN- β antigenically and in pH stability (labile at pH 2). IFN- γ appears to be more effective as an immunoregulator, and thus may possibly be more important in cancer treatment. It also appears to potentiate the antiviral effect of α and β IFNs 5-10 fold. There seems to be a synergistic amplification of interferon-mediated protection against viral infection [9]. Studying autoimmune disease, Dr. ABNER NOTKINS has observed that immune interferon is present in the sera of 46% of patients with systemic lupus erythematosus, 50% with rheumatoid arthritis, 60% with scleroderma, and 27% with Sjogren syndrome. Serial serum samples showed a good correlation between interferon titres and disease activity. It is not known whether immune interferon contributes to the autoimmune disease process and/or protects the host from viral infections.

If these clinical studies in cancer patients and against infectious diseases continue to show beneficial effects, the biological systems currently being used to produce interferon will not suffice. Estimates of future interferon needs have led to considerable activity in purification of the active moiety of interferon in order to determine its amino acid sequence so that eventual synthesis

will be possible. Other studies utilizing DNA recombinant and cloning techniques are progressing at a rapid rate, and bacterial organisms are genetically engineered to produce human interferon. Without success in these production areas, demonstration of efficacy will not have much impact in clinical medicine.

Amantadine

Although amantadine was approved for the prevention of influenza A (H2N2) in the U.S. as early as 1966, it was not widely accepted. This was due to a variety of factors, one of which was concern about side effects. In 1976, on the strength of the data accumulated on side effects from its use in thousands of Parkinson's disease patients, the threat of swine flu, and animal efficacy studies, amantadine was licensed for use against all influenza A viruses. Still, there was a reluctance to prescribe it. A number of studies were performed to investigate again the efficacy and safety of the drug. During the initial outbreak of H1N1 influenza in 1978-79, a double-blind, placebo-controlled trial in 286 college students was performed by Dr. ARNOLD MONTO. The study [10] showed an efficacy rate of 70.7% in prevention of laboratory-confirmed clinical influenza. Equally important were the data on side effects. It became clear that if side effects are to be observed, they occur in the first 48 h of treatment or not at all. These symptoms are mild, and generally include insomnia, lightheadedness or dizziness, difficulty in concentration or drowsiness. The withdrawal rate in this study was 6.2%.

Studies were also performed by Dr. GORDON DOUGLAS on the therapeutic value of amantadine [11]. When the drug was administered in established influenza A infection, there was an accelerated improvement of clinical illness and pulmonary physiologic dysfunction. A more recent study [12] has compared the therapeutic efficacy of amantadine and rimantadine in naturally occurring influenza. Forty-five university students with proven H1N1 influenza A were randomly treated with either amantadine (14 students), rimantadine (19 students), or placebo (12 students). Improvement in the amantadine group was observed within 24 h; both the amantadine and rimantadine groups showed significant improvement at 48 h. Both drug treated groups returned to class earlier, required less aspirin, and shed less virus than the placebo-treated group. In this study, both drugs were effective, but amantadine exerted a more rapid effect and was felt to be the drug of choice by the investigators.

Based on these and similar studies, amantadine has been given a new vote of confidence [13] in the U.S. Studies on the relative merits of amantadine and rimantadine are continuing.

Ribavirin

Ribavirin is a synthetic nucleoside that has exhibited *in vitro* and *in vivo* antiviral activity against both type A and B influenza viruses. Clinical studies have yielded conflicting results. Recently, a collaborative study was performed to determine the role of the drug in influenza [14]. The double-blind, placebo-controlled study included 97 young adult males naturally infected with influenza A (H1N1) in the winter of 1978-79. The drug was administered orally for five days beginning within the first 48 h of clinical illness. The dosage used was 1000 mg/day. There were no differences in clinical signs and symptoms, nor in virus shedding, between the drug and placebo groups. Significant rises in bilirubin and reticulocyte counts were observed in the ribovirin group. This study should provide the final answer to the role of this drug against influenza: it has none.

The drug, however, does appear to have a beneficial effect against arenavirus infections in animals. It has been tested against Lassa fever virus, Bolivian haemorrhagic fever (Machupo virus) and Pichinde virus. Following administration intramuscularly to Rhesus monkeys infected with Lassa virus, there was a decrease in viraemia and enhanced survival. Similar results were obtained with viraemia in Machupo virus infection. Ribavirin did not, however, prevent the late neurologic syndrome which was fatal to the animals (E. STEPHEN, personal communication). Based on these animal studies, some Lassa fever patients in Africa were treated with ribavirin, but no effect was observed. This is believed to have been due to the low dose administered, the route used (oral instead of systemic) and the advanced stage of disease. Studies are planned using the drug at a higher intravenous dose in such patients (K. JOHNSON, personal communication). It is too early to predict, but it does appear that, unlike influenza, ribavirin may be useful against arenaviruses.

Adenine arabinoside

A compound of considerable interest is adenine arabinoside (ara-A), the first agent to be approved in the United States for systemic treatment of an ongoing serious virus infection. Ara-A, 9- β -D-arabinofuranosyl-adenine, is a purine nucleoside with *in vitro* and *in vivo* antiviral activity against herpes simplex, varicella, cytomegalo and vaccinia viruses. The mechanism of action is not fully understood; it appears to selectively inhibit viral DNA synthesis.

In 1972, having reviewed the current state of the art, the Antiviral Substances Program of the NIAID decided that in addition to interferon the compound that seemed to hold the greatest promise for clinical application was ara-A. A Collaborative Antiviral Study Group was established and four double-blind, placebo-controlled protocols were developed, (i) herpes ence-

phalitis, (ii) early-onset varicella-zoster virus infections in immunocompromised patients, (iii) progressive mucocutaneous herpes simplex virus infections in immunocompromised patients, and (iv) neonatal herpes simplex infection. In 1977, the results of the encephalitis study clearly demonstrated the efficacy of the drug. Mortality due to biopsy-proven herpes simplex encephalitis was 70%, whereas treatment with ara-A reduced it to 28%. Although there are neurological sequelae in some survivors, these can be limited if the patient is treated early in the infection [15]. The prognosis is poor if treatment is initiated when the patient is comatose. This study led to the approval of ara-A by the U.S.-FDA for this disease. Although the numbers in the original report were small, further study in over 100 patients has supported the original finding.

The neonatal herpes simplex protocol was recently analysed [16] and a beneficial effect was demonstrated. Fifty-six infected newborns were studied with comparable distribution in the placebo and drug groups. Overall mortality was significantly reduced in babies with CNS and disseminated disease from 74% to 38% with drug therapy. The outcome with disseminated disease was not as good as with localized CNS disease. The mortality in the former was reduced from 85% to 57%, whereas in the latter it was reduced from 50% to 10%. Mortality was uncommonly associated with localized skin, eye or mouth infections; however, severe sequelae occurred in 38% of placebo recipients but not in those on drug therapy. The drug was administered intravenously at a dose of 15 mg/kg/day over a 12 h period for ten days, similar to the encephalitis study.

The code for the zoster study will be broken this summer. An earlier crossover study in which patients were treated for five days with either drug or placebo and then followed for an additional five days with the opposite treatment, showed promise. Because of the design of the study, only early symptoms could be assessed. Virus titre, cessation of new vesicle formation, total pustulation, and cessation of pain all favoured the drug group.

The mucocutaneous protocol will probably be decoded at the end of the year. One of the problems with ara-A is its poor solubility. It must be administered by slow drip over a 12 h period in order to achieve the dosage required. The failure of ara-A, applied as an ointment, to benefit mucocutaneous herpes (fever blisters) or genital herpes, is attributed to its relative insolubility. It is, however, effective as a topical treatment for herpetic keratitis. A more soluble form of the drug, adenine arabinoside monophosphate, is currently under study. NIAID-supported studies have shown that the monophosphate is not effective when applied topically [17]; when given systemically, however, it is rapidly converted to ara-A, and it is expected that it will be as efficacious as the parent compound. Clinical studies to compare it to ara-A in the treatment of encephalitis have been initiated. Protocols for neonatal herpes are being

developed. The reduction of the fluid volume required for administration of ara-A will be of definite benefit to the patient.

Early clinical studies testing ara-A for efficacy in cytomegalovirus infection (bone marrow and renal transplant patients) have not been promising. Although little toxicity was observed in the herpes simplex studies, there is some indication that more serious toxicity may occur in patients with renal or liver impairment.

Acyclovir

One of the more promising new anti-herpes compounds is 9-(2-hydroxy-ethoxymethyl) guanine, acyclovir. This compound, developed by Burroughs-Wellcome Research Laboratories was found to have marked antiviral activity in animal models of herpes virus infections and to be associated with very low toxicity. It is highly specific; it appears to be phosphorylated to a monophosphate by a herpesvirus-specified thymidine kinase, whereas this does not readily occur in uninfected cells. This virus specificity is deduced because in herpes-infected cells the monophosphate is converted to triphosphate to a much greater extent than in uninfected cells [18]. Trials testing this compound for topical treatment of herpes labialis and genital herpes are under way. Preliminary data from both studies indicate some measure of success. However, further study and analysis is needed for definitive results. Phase one studies on pharmacology-toxicology of the drug for systemic application have been completed, and clinical studies are currently being initiated to compare it to ara-A and ara-AMP for treatment of biopsy-proven herpes encephalitis. It is our intent to initiate similar studies with acyclovir for neonatal herpes and herpes zoster.

Other antivirals

Phosphonoacetic acid caused considerable stir and interest when it was shown to be very effective against herpes viruses. When it, however, was found to be retained by the bone, interest lagged due to its potential toxicity. Studies continued in an effort to find an analogue which had antiviral efficacy but less toxic potential. Phosphonoformate inhibits the DNA polymerase of herpes virus and, thereby, viral replication. It does not affect cellular DNA synthesis or cell proliferation unless high concentrations are used. *In vivo* studies show promise for this compound, but problems with bone retention still need further study. Attempts at finding more suitable analogues are continuing.

Arildone (4-[6-(2-chloro-4-methoxy)phenoxy]hexyl]-3,5-heptanedione), identified by STERLING-WINTHROP, represents a new class of antiviral drug which selectively inhibit the replication of some RNA and DNA viruses (herpes virus types 1 and 2, varicella-zoster, poliovirus types II and III, ECHO viruses

9 and 11, rhinoviruses types 2, 14, and 17, equine rhinovirus and respiratory syncytial virus). Although the exact mechanism of action is not known, it is believed to inhibit virus uncoating. It does not inhibit viral adsorption or penetration nor is it virucidal [19]. Studies *in vivo* have indicated that it does have some efficacy against herpes 1 and 2 in the guinea pig when applied topically (PAUL CAME, personal communication).

Another compound of interest is E-5-(2-bromovinyl)-2'-deoxyuridine which has a selective activity against herpes simplex viruses 1 and 2, particularly type 1, and varicella-zoster. [20]. One of the most interesting aspects is its efficacy against varicella-zoster. This drug is in its early stages of development and it is too early to determine its clinical potential.

The value of a true double-blind, placebo controlled clinical study in evaluating the efficacy of antiviral drugs cannot be overemphasized. It is only in this manner that a compound can be evaluated efficiently in a relatively brief period of time. Historic controls are not satisfactory. It was not until such double-blind studies were done that the lack of efficacy and harmful effects of systemic cytosine arabinoside (against zoster) and IDU (against herpes encephalitis) were clearly demonstrated. Although predictive, animal studies cannot accurately measure efficacy or drug toxicity in humans.

In conclusion, it appears that the hope and expectations of the 60s have come to fruition. The sceptics have partially been silenced. We seem to be in the early log phase of research and clinical application of antivirals. It is hoped that the 80s will completely silence the sceptics and yield many more clinically useful agents for the treatment of viral disease and cancer.

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EFFECT OF IMMUNOSTIMULATING AGENTS ON VIRAL INFECTIONS*

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At present, many severe viral diseases that affect humans and domestic animals can be efficiently prevented by the use of appropriate vaccines. As far as vaccines for humans are concerned, the best example is represented by the smallpox vaccination campaign organized by the WHO that ended up in the practical eradication of the disease in the whole world. Vaccination against smallpox, influenza, mumps, measles, rubella, rabies, yellow fever, hepatitis B has been extensively adopted to spare from such diseases large human populations or individuals presenting a high risk of infection. For other viruses (adenoviruses, respiratory syncytial virus, cytomegalovirus and varicella virus) vaccination may become a reality before long. Despite all the progress reported so far, man is still susceptible to a multitude of viruses (Table I) for which it is unlikely that specific vaccines will be developed. An alternative approach would be the development of chemotherapeutic antiviral agents. So far, however, very little progress has been reported in this field (amantadine, methisazone, vidarabine, idoxuridine) despite many years of active work, mostly carried out by pharmaceutical companies.

Although chemotherapy research will be facilitated in the future by the progressing knowledge of the molecular biology of viral replication, it is unlikely that much effort will be put in the development of drugs which could be used against those viral infections characterized by a low incidence (e.g. Marburg) or by symptoms (e.g. respiratory, enteric infections) which are common to diseases caused also by other viral or nonviral pathogens. Most probably, nonspecific antiviral agents would be needed for such infections. An example of a broad active antiviral agent is the interferon molecule(s).

Interferon is presently the object of extensive investigations in academic as well as in pharmaceutical research. Its application in antiviral chemotherapy is controversial: it is hoped that the modest results obtained so far [1] can be improved with the availability of larger amounts of very pure interferon preparations.

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Table I

Viruses which may be considered targets for chemotherapy or/and immunotherapy

Family	Genus	Species or serotype (s)	Human disease-infection
<i>Picornaviridae</i>	<i>Enterovirus</i>	Coxsackie A (23s) Coxsackie B (6s) Echoviruses (31s)	Respiratory Diarrhoea, meningitis Common cold
	<i>Rhinoviruses</i>	>100 serotypes	
<i>Herpetoviridae</i>	<i>Herpesvirus</i>	{Herpes simplex type 1} {Herpes simplex type 2} Varicella, zoster Cytomegalovirus	Dermatological encephali- tis Varicella, herpes zoster— Hepatitis
<i>Paramyxoviridae</i>	<i>Paramyxovirus</i>	Parainfluenza 1 Parainfluenza 2 – 5	Respiratory
	<i>Morbillivirus</i>	Measles virus	Subacute sclerosing encephalitis
<i>Rhabdoviridae</i>	<i>Vesiculovirus</i>	Vesicular stomatitis	Respiratory
<i>Arenaviridae</i>	<i>Arenavirus</i>	Lymphocytic choriome- ningitis virus	Meningitis
		Lassa virus	Lassa fever
<i>Papovaviridae</i>	<i>Papilloma virus</i>	Human wart virus	Wart
	<i>Polyoma virus</i>	JC virus	Progressive multifocal leukoencephalopathy
<i>Coronaviridae</i>	<i>Coronavirus</i>	B814, 229E, OC43	Respiratory
<i>Togaviridae</i>	<i>Alphavirus</i>	Eastern } equine Western } encephalitis Venezuelan }	Haemorrhagic fever
	<i>Flavivirus</i>	Dengue (4 s)	Fever, arthritis
Unclassified		Marburg	} Haemorrhagic fever Hepatitis Creutzfeld-Jakob
		Ebola	
		Hepatitis A	
		—	

For the past 10 years, scientists have considered the possibility of increasing, by administration of products, the natural immunological defenses of the host in order to protect him from infectious diseases as well as from tumour invasion. Since common immunological mechanisms are, up to a certain extent, at the basis of the resistance to infections caused by different viruses, it would not be too unrealistic to think that a drug capable of enhancing such mechanisms would possess activity toward many unrelated viruses. On the other hand, there is strong evidence that ordinary mild or asymptomatic infections in normal people, can produce severe and often lethal diseases in individuals, whose immunosystem has been impaired either naturally (congenital and acquired immunodeficiencies) or as the result of drug administration. The increasing use in medical practice of cytotoxic drugs for the treat-

Table II
Potential target diseases for immunotherapy

Disease	Examples inosiplex (I) or levamisole (L) exerting activity on the
<i>Primary immunodeficiencies ?</i>	
<i>Secondary immunodeficiencies</i>	
<i>Chemotherapy:</i> steroids, cytotoxic agents	
<i>Virus infections:</i> measles, CMV, influenza rhinovirus HSV-1	(I) modulation PHA response [6] (I) modulation PHA response [7] (I) modulation PHA response [8] (L) disease [9]
<i>Malignant diseases:</i> leukaemia, Hodgkin disease non-Hodgkin lymphoma multiple myeloma	
<i>Age related deficiency:</i> newborn, elderly individuals	
<i>Persistent viral infections:</i> rubella, CMV, EBV, progressive multifocal leukoencephalopathy hepatitis B subacute sclerosing panencephalitis wart	(L) disease [10] (I) disease [11] (L) disease [12]
<i>Inflammatory diseases</i> circulating immunocomplexes	
<i>Common but non-severe forms of viral infections</i>	(L) disease-unknown aetiology [13]

ment of cancer patients or those patients undergoing transplantation is creating more individuals with weakened immunological defences. For example, in transplanted patients a high risk exists for the development of primary cytomegalovirus (CMV) infection or reactivation of latent virus.

In these cases, both the allograft reaction itself [2] and treatment with cytotoxic drugs (methotrexate, azathioprine and cyclophosphamide) play an important role [3].

Although herpes simplex type 1 and type 2 viral infections are common about 1 month after kidney transplantation, they occur at a lower extent than those caused by CMV [4]. Chickenpox, which is a benign disease in normal children, can become very severe and sometimes lethal in children affected by leukaemia [5]. In the well recognized forms of immunological deficiencies quoted above, the use of an immunostimulating drug could reduce the severity and the frequency of the viral disease. In addition, other diseases may be considered as potential targets for immunotherapy (Table II). In the following section we

shall discuss 4 examples of immunostimulating agents: mycobacteria, *Corynebacterium parvum*, inosiplex and levamisole. We shall present mostly our personal laboratory experience with these products; a comprehensive review on the topic of immunostimulating substances with antiviral activity has recently been published [14].

Mycobacteria and mycobacterial products

Following the pioneer clinical studies of MATHE's group [15], live bacillus Calmette-Guérin (BCG) is quite often used today for cancer immunotherapy in man. As to experimental viral infections, intravenous administration of BCG into mice at least 8 days before challenge (intravenous, intraperitoneal or subcutaneous) with Mengovirus has been shown to exert a marked protective activity [16]. Similar results were obtained in the case of i.p. infection of newborn mice with HSV-2 [17]; BCG alone, however, failed to protect mice from intravaginal challenge with the same virus unless it was applied in combination with passive anti HSV-2 immunization [18].

Our group [19] has reported that i.v. administration of BCG to mice between 15 and 31 days before challenge with encephalomyocarditis (EMC) virus, murine hepatitis virus (MHV), HSV-1, HSV-2, foot and mouth disease virus (FMDV) and influenza virus resulted in a significant decrease of mortality, from an overall 82.2% in the control group to 58.7% in the treated group. Enhanced resistance to AO influenza virus infection in mice has been also found in the case of intranasal administration of BCG [20].

A striking increase in the virus (EMC) LD₅₀ has been reported to occur in mice treated one month before the infection with a nonviable preparation of emulsified *Mycobacterium tuberculosis* in mineral oil [21, 22]. The increased resistance was not associated with the development of anti-EMC antibodies or the presence of an intact thymus. Since it was abrogated by silica or cyclophosphamide treatment given shortly before the infection, the authors suggested that activation of macrophages was the mechanism responsible of the protective effect of the emulsion. Macrophages play a very important role in the resistance to viral infections [23], probably through the participation of many mechanisms (Table III). Furthermore it is known that the most powerful immunostimulants (e.g. BCG, *C. parvum*) induce the formation of long lasting granulomata [24].

One may wonder whether any inflammatory process going on in an animal could lead to an increased resistance to pathogens. For example, a sterile subcutaneous granuloma induced in mice by inoculation of a sterile irritant (Talcum powder in a gel of Ca phosphate-TPC) has been shown to markedly protect them from bacteria, fungi, protozoal infections as well as from tumour invasion [25].

Table III*Macrophage mediated events in the resistance to virus infections*

Phagocytosis
Interferon production
Restriction of intrinsic virus replication
Restriction of extrinsic virus replication
Cytotoxicity for virus infected cells
Production of components of the complement system
Participation in inflammation
Antigen processing
Regulation of lymphocyte activities

We were interested in examining whether mice bearing a TPC induced granuloma developed a higher resistance to viruses. We found, however, that such mice were just as sensitive as control mice to infection with EMC virus, MHV or HSV-1 [26]. The reasons for which the TPC-induced granuloma fails to protect mice from viral infections, while it can render them resistant to a variety of nonviral pathogens, are unclear. One explanation could be that the granuloma-activated macrophages observed by FAUVE [25] are not sufficiently activated to lead to a state of higher antiviral resistance. On the other hand interferon, which is not induced by the TPC granuloma, might be an essential component of the increased antiviral resistance after treatment with other inflammatory substances (e.g. BCG, *C. parvum*).

Mycobacteria derived products possessing general immunostimulating properties, have been described: MER (methanol extraction residue), cord factor, MDP (*N*-acetyl muramyl-L-alanyl-D-isoglutamine). There is, however, no information on their activity toward viral infection. WERNER *et al.* [27] have reported that a polysaccharide-peptidoglycan fraction (15 000–30 000 daltons) isolated from mycobacteria exerted an enhancing effect on the resistance of mice to EMC virus.

Corynebacteria

Clinical studies carried out in several oncologic centres throughout the world have suggested that killed preparations of *C. parvum* can give favourable results in cancer immunotherapy.

Injection of *C. parvum* into mice has been shown to enhance their resistance to a variety of viral infections such as EMC [28], HSV-1 [29], murine CMV and Semliki Forest [30]. As in the case of BCG, the protection occurs when *C. parvum* is inoculated usually seven days before challenge. Still in the case of infection of newborn mice with Junin virus [31], activity was optimum when *C. parvum* was administered 3 days after infection.

Among the immunological effects induced by *C. parvum* (Table IV) macrophage activation does not seem to play a major role in the resistance of

Table IV

Effects of C. parvum on the immune system

Increase in antibody production
 Activation of the classical and alternate complement pathway
 Decrease in cell mediated immunity
 e.g. delayed hypersensitivity,
 graft-versus-host reaction
 Macrophage activation
 Bone marrow macrophage colony production
 Interferon production
 NK activity

mice to Junin virus, since depression of the mononuclear phagocytic system with silica does not aggravate the infection. It is likely that the therapeutic effect of *C. parvum* in the case of the Junin virus is due to a decrease of T lymphocyte response following virus entry.

In contrast, the activity of *C. parvum* in the case of other viruses (e.g. HSV) has been shown, on the basis of cell transfer experiments, to be mediated to a large extent by macrophages [30].

In our laboratory, treatment of mice with *C. parvum* 7 days before challenge with HSV-1 increased 5–10 fold the virus LD₅₀. We were interested to see whether peritoneal macrophages obtained from mice treated 7 days earlier with *C. parvum* were less permissive *in vitro* to HSV-1 replication than normal resident macrophages. In a series of experiments we have compared, after *in vitro* infection, the two cellular populations for: (a) the number of infectious centres (Table V), (b) their capacity, following injection, to kill recipient normal mice (Table VI).

Table V

HSV-1 infectious centres in macrophages

Experiment No	Virus input (HSV ₁ PFU/monolayer)				Source of cells mice treated with
	5760	1920	720	360	
1	>30	20.5	9.7	—	<i>C. parvum</i> saline
	>30	23	14.2	—	
2	—	—	—	1.5	<i>C. parvum</i> saline
	—	—	—	3.5	
3	—	—	7.5	1.0	<i>C. parvum</i> saline
	—	—	15.5	3.5	

Mice (5-week-old female OF₁) were treated i.p. with *C. parvum* (10 mg/kg) or saline. Seven days later the animals were killed and their peritoneal cells were collected. Cells were cultured for 3 h in Limbro-Multiwell plates (1 × 10⁶ cells per well in M 199, 10% SVF medium). Monolayers were then washed and infected for 1 h with HSV-1 at different dilutions. After washing, 2 × 10⁶ OF₁ embryo fibroblasts, in Earle's BME containing 2% FCS and 0.75% carboxymethylcellulose, were added to each well. Plaques (infectious centres) were counted after 72 h

Table VI

Infection of mice by transfer of HSV-1 (infected) peritoneal cells

M.O.I.	Source of cells Mice treated with	Number of surviving mice/number of mice injected
0.1	<i>C. parvum</i> saline	0/6
		0/2
0.02	<i>C. parvum</i> saline	0/7
		0/4
0.01	<i>C. parvum</i> saline	0/4
		0/5
0.005	<i>C. parvum</i> saline	1/3
		1/3
0	<i>C. parvum</i> saline	3/3
		4/4

Mice (5-week-old, female OF₁) were treated i.p. with *C. parvum* (10 mg/kg) or saline. Seven days later the animals were killed and their peritoneal cells were collected. Cells were infected (1 h, 37 °C) in siliconized tubes with HSV-1 at different MOIs. After washing 1.8×10^6 viable cells in 0.3 ml Hanks' BSS were injected i.p. to recipient normal mice (OF₁). Mortality was recorded.

The results showed that macrophages of *C. parvum* treated mice do not differ significantly from normal macrophages in their intrinsic antiviral activity as well as in their capacity to spread the infection *in vivo*.

Thus in the case of HSV-1 infection the high *in vivo* resistance observed in *C. parvum* treated mice does not correlate with an enhanced intrinsic antiviral activity of their macrophages.

It is worth mentioning that *C. parvum* activated macrophages can markedly inhibit the yield of virus in HSV-2 infected cells [32, 33].

Although the mechanism of such "extrinsic antiviral activity" is unknown, it might account to a large extent for the activity of *C. parvum* toward such virus.

Inosiplex

Inosiplex (Isoprinosine®), a mixture or "complex" of *p*-acetamidobenzoic acid, 1-dimethylamino-2-propanol and inosine (3 : 3 : 1 molar ratio), is a drug which has been used in several countries as a nontoxic antiviral agent. Its antiviral activity is, however, limited to a moderate inhibition of rhinovirus and influenza virus (A2) *in vitro* [34] and, with the exception of the fibroma virus infection in rabbits, no significant activity has been found *in vivo* [35]. Later on, it has been suggested that the drug's antiviral activity was due to its stimulation of the immune system. *In vitro*, inosiplex potentiates lymphocytic

responses such as antibody formation [34] and the mitogenic response to phytohaemagglutinin (PHA) [36, 37].

We have found that mouse peritoneal macrophages incubated with inosiplex for 18 h present a significantly higher capacity, than control macrophages, to phagocyte opsonised (antibody-treated) sheep erythrocytes (Fig. 1).

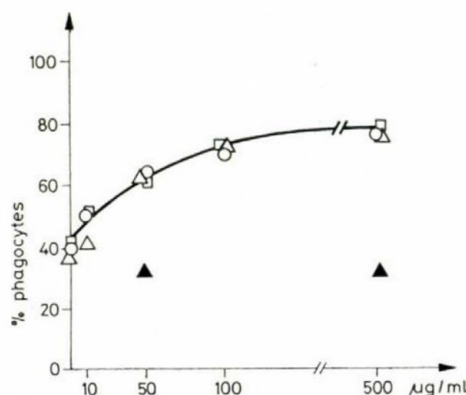


Fig. 1. Resident mouse peritoneal macrophages were cultured for 18 h with different concentrations of inosiplex. The drug was washed away and sheep erythrocytes opsonized with a sub-optimal concentration of antibodies were added. Phagocytosis was allowed to proceed for 60 min and the number of cells containing at least 2 sheep erythrocytes (phagocytes) was counted among the whole population. Results are expressed as phagocytes in a total of 100 cells. ○ □ △ 3 experiments; ▲ inosiplex added with the sheep erythrocytes (time 0)

Table VII

Influence of inosiplex constituents on the phagocytic activity of macrophages

Substance	Phagocytes, per cent	
	exp. 1	exp. 2
M ∅ control	61	56
Inosiplex	87	93
A	72	79
B	61	51
C	71	68
A+B	79	83
B+C	72	66
A+C	78	79
A+B+C	86	90

Resident mouse peritoneal macrophages were cultured for 18 h in the presence of inosiplex (0.45 mM) or its constituents: A inosine (0.45 mM), B p-acetamidobenzoate (1.35 mM), C N,N-dimethylamino-2-propanol (1.35 mM) either alone or in combination. Phagocytosis was carried out as in Fig. 1

No effect is obtained if the drug is added to control macrophages during the phagocytosis process; thus it seems that a certain amount of time is necessary for inosiplex to exert a stimulation of macrophages.

Maximum stimulation of phagocytosis was found to occur when all the three components were added together to the macrophage cultures, although inosine or dimethylaminopropanol alone also exerted a significant activity (Table VII). Inosiplex has been found to increase the resistance of mice to influenza [34, 37, 38] and herpes simplex [39] virus infection and to potentiate the interferon activity against EMC virus [40].

In our laboratory, administration of inosiplex to mice in drinking water (1%) soon after infection with MHV, HSV-1, influenza virus, EMC virus has sometimes increased their survival time. Still, no significant reduction in the mortality or in the virus titre in the lungs has been observed in the case of influenza infection. Inosiplex can modulate some parameters of the immune system, such as the response of lymphocytes to PHA, which have been perturbed in some viral infections (see Table II).

Levamisole

Following the observation of RENOUX and RENOUX [41] that the anti-helminthic drug levamisole increased the protective activity of a *Brucella* vaccine in mice, extensive research has been carried out on its immunostimulating properties. The wide interest in such a drug was easy to understand.

As compared to others, levamisole was the first immunostimulating agent to be described, with a chemically defined structure and devoid of major toxicologic effects. Very little work, however, has been done with levamisole on the viral infections of laboratory animals. The only positive result has been obtained with the HSV-2 infection of suckling rats [42, 43], where the treated group displayed a lower mortality than the control one. In contrast, levamisole did not exert a significant activity on i.p. or i.v. infection of newborn or adult

Table VIII
Effect of levamisole on HSV-1 infection

Dose (mg/kg)	Surviving mice	Increase in mean survival time, per cent
0	0/20	100
2.5	4/20	147 $P = 0.005$
5	2/20	122 [N. S.]

Male, 5-week-old Balb/C mice were treated i.p. with levamisole or saline 24 h before i.p. infection with HSV-1

mice with HSV-2 [17, 44]. In our laboratory, we have studied the therapeutic effect of levamisole in mice infected with influenza virus, MHV, HSV-1, HSV-2 and EMC virus. With the exception of a slight increase in survival in the case of MHV or influenza A2 virus, administration of levamisole in drinking water from day 0 throughout the experiments failed to protect mice from such acute diseases. We found, however, that mice (Balb/C) treated i.p. with levamisole 24 h before i.p. infection with HSV-1 presented a longer survival than control (saline) treated mice (Table VIII). Induction of low levels of interferon by levamisole [45] might account for the positive results obtained in such experiments.

Discussion

Two important points arise from the fragmentary results presented. The first one deals with the mechanism of the antiviral activity of immunostimulating agents. In the case of BCG and *C. parvum* it is likely that their marked antiviral activity originates from the fact that they induce strong inflammatory and delayed type hypersensitivity (DTH) reactions. Their immunogenic nature and the fact that they can persist for a long time in the organism contribute to the high level of both cellular responses. Inoculation of these agents provokes the formation of granulomatous reactions and the release of mediators of inflammation. A partial activation of macrophages occurs as the result of the inflammatory reaction itself. For example *C. parvum* added *in vitro* can increase in normal macrophages their cytotoxic potential and their enzyme content [46-48], although the presence of immune spleen cells can greatly amplify such reactions. Killed mycobacteria enhance the resistance of thymectomized mice to EMC virus [22]. Furthermore *C. parvum* can protect normal mice from EMC infection but not from HSV, when administered 24 h before challenge, thus long before the development of a cell mediated immunity to *C. parvum* (ZERIAL *et al.*, unpublished results). The antiviral state, however, is not induced by all kinds of persistent inflammatory reactions. For example, mice in which a sterile granuloma was induced by s.c. implantation of talcum powder (TPC) do not exhibit a higher resistance to MHV, EMC and HSV-1, while they are fully protected from infections with a variety of nonviral pathogens.

This suggests that the primary process of macrophage activation, the one occurring as a result of the inflammatory reaction itself, is not sufficient to build up a strong antiviral state. While progression of the inflammatory reaction occurs, so does lymphocyte sensitization. Production of soluble mediators by lymphocytes, further increases the activation of macrophages [46, 48] and perhaps they also contribute to the increase of inflammation. Among the lymphokines produced, type II "immune" interferon must play a major

role in limiting the spread of infection, either by itself or through activation of the natural killer (NK) cell system [49]. Obviously, since DTH reactions are absent in mice bearing the TPC-induced granuloma, a further activation of macrophages and the participation of the interferon system do not occur in this situation. The role played by activated macrophages in the increased antiviral resistance of mice treated with *C. parvum* is unclear. Our results suggest that macrophages from *C. parvum* immune mice do not possess a higher virucidal activity toward HSV (intrinsic antiviral activity).

Extrinsic antiviral activity could be responsible for the protective effect of macrophages *in vivo*, as seen after their transfer [33]. In the case of levamisole and inosiplex, which do not manifest strong antiviral activities in experimental infections, it is more difficult to understand the mechanism underlying some encouraging results which have been observed in clinical studies.

Interferon production by levamisole [45] or increase of the antiviral activity of interferon by inosiplex [40] are only some of the many immunological responses which are induced by these drugs.

The possibility that these agents are active because they enhance immunological functions which have been impaired, e.g. by viral infections, is worth being investigated. This brings us to the second point of the discussion, namely the poor results which are obtained with levamisole or inosiplex in laboratory animals.

When some antiviral activity is noticed, it is limited within a narrow dose range. Furthermore the activity might affect a specific viral system only and/or a particular strain of mice.

Further studies are needed to develop adequate infectious systems in laboratory animals in order to assess the antiviral activity of such kind of immunomodulators. Particular emphasis should be given to those viral non-lethal diseases which occur with high frequency in man and domestic animals (e. g. respiratory infection). Some models have already been described. For example animals like ferrets [50] and hamsters [51] infected with influenza virus develop clinical responses similar to those seen in man.

Since immunotherapy has provided in a few clinical trials some beneficial effects in immunodepressed patients it would be worthwhile to set up adequate models of immunodeficiency (e.g. following cytotoxic therapy) in small laboratory animals.

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INDEX

<i>Lantos, J., Fekete, J., Király, K.</i> : R-plasmid Study of an Outbreak Caused by Multi-resistant Strains of <i>Salmonella panama</i>	211
<i>Ádám, Ě., Nász, I.</i> : Mutual Orientation of Adenovirus Hexon Polypeptides in a Two-Dimensional Crystalline Array	219
<i>Šula, L.</i> : The Colonial Microtexture of Human Mycobacterial Strains Isolated in Burma	229
<i>Nyerges, G., Jaszovszky, I.</i> : Reliability of the Rabbit Pyrogen Test and of the Limulus Test in Predicting the Pyrogenicity of Vaccines in Man	235
<i>Chautard, P., Guiraud, J. P., Galzy, P.</i> : Inulinase Activity of <i>Pichia polymorpha</i>	245
<i>Billiau, A.</i> : Pharmacokinetic and Pharmacological Aspects of Interferon Therapy in Man	257
<i>Bodo, G.</i> : Large-Scale Production and Purification of Human Lymphoblastoid Interferon	263
<i>Borecký, L., Hajnická, V., Kontsek, P., Lackovič, V., Russ, G., Pěkníková, J., Čapková, J., Rajčáni, J., Fuchsberger, N.</i> : Growth Inhibitory Effect of Mouse Interferon in Transformed Cells	271
<i>Burke, D. C.</i> : Some Recent Advances in the Molecular Biology of the Interferon System	283
<i>de Clercq, E.</i> : Nucleoside Analogues as Antiviral Agents	289
<i>de Clercq, E., Verhelst, G., Descamps, J., Bergstrom, D. E.</i> : Differential Inhibition of Herpes Simplex Viruses, Type 1 (HSV-1) and Type 2 (HSV-2), by (E)-5-(2-X-vinyl)-2'-deoxyuridines	307
<i>Galasso, G. J.</i> : Antiviral Agents: the Road from Scepticism to Efficacy	313
<i>Zerial, A., Werner, G. H.</i> : Effect of Immunostimulating Agents on Viral Infections	325

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PROTOPLAST FUSION IN THE YEAST *CANDIDA UTILIS*

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Protoplasts obtained by snail enzyme treatment from two stable auxotrophic mutants of the yeast *Candida utilis* were induced to fuse by the use of polyethylene glycol. The hybrids formed from the auxotrophic parental strains were selected by complementation on stabilized minimal medium. Many of the hybrids were unstable and readily dissociated into their parental strains. Others, in which parental nuclei had fused, gave stable hybrid progeny. Cytological and genetic evidence of these processes is presented.

Some basic genetic studies on the yeast *Candida utilis* have recently been made [1–3]. In these works the authors reported the isolation of auxotrophic mutants and complementation between them. They suggested the existence of a diploid stage or a dikaryon phase in this yeast.

More recently, DELGADO *et al.* [4] presented quantitative results concerning factors which influence hybrid formation between double auxotrophic mutants of *C. utilis*.

In this paper we report the use of polyethylene glycol (PEG) to induce hybrid formation by protoplast fusion from two stable auxotrophic mutants of *C. utilis*. The results presented show that not only does fusion of protoplasts take place, but that this process is followed by nuclear fusion leading to stable diploids.

Materials and methods

Strains. Double auxotrophic mutants VN9–9 (*cys*₁ and/or *met*₁, *ura*₁) and VN5–15 (*ade*₁, *met*₂) were obtained in our laboratory from the original strain Y-900 (a gift of P. S. Dawson from the National Research Council of Canada) using *N*-methyl-*N*'-nitro-*N*-nitrosoguanidine as the mutagenic agent, followed by a snail enzyme enrichment procedure [3].

Media and chemicals. Yeast extract peptone medium (YPD) consisted of (per litre): yeast extract, 10 g; peptone, 10 g; dextrose, 20 g. Minimal medium (MM) was prepared according to GALZY and SŁONIMSKI [5]. When nucleic acid bases and amino acids were used for supplementation, a sterile solution containing adenine, methionine, cysteine, and uracil was made up to a final concentration of 10 mg of each per litre of medium. For solid media 20 g agar Oxoid No. 3 was added per litre. MMM consisted of MM stabilized with 0.7 M mannitol. Sporulation medium consisted of potassium acetate, 0.1 M; glucose, 0.2%; malt extract, 0.2%. Pretreatment medium (PTM) contained 100 mM Tris, 5 mM dithiothreitol and 5 mM EDTA-Na₂ pH 8.

Protoplast formation. A modification of the method used by HOUSET *et al.* [6] for *Schizosaccharomyces pombe* was applied. The cells were collected in the log-phase by centrifugation, washed twice with sterile water and resuspended in PTM (5×10^8 cells/ml). The suspensions were shaken at 30 °C for 10 min, washed twice with 0.6 M sorbitol, and resuspended in a mixed

solution of 0.1 ml snail enzyme (Industrie Biologique Française; Gennevilliers) and 1 ml 0.7 M mannitol. The suspension was incubated at 30 °C with shaking. The production of protoplasts was followed by phase contrast microscopy and checked for osmotic sensitivity.

Fusion process. Protoplast suspensions of both auxotrophic mutants, containing 5×10^7 protoplasts per ml each, were mixed in 1:1 ratio and centrifuged at 1000 g for 20 min. After centrifugation the supernatant was discarded and the protoplasts were suspended in 2 ml 35% PEG (MW 6000) in 10 mM CaCl_2 and gently shaken. After 1 h of incubation the protoplasts were embedded in MMM at 42 °C and transferred as a thin layer to the surface of MMM plates which were subsequently incubated at 30 °C for 10 days. The colonies, which presumably arose by fusion, were isolated and transferred into YPD and MM medium. Protoplasts from both auxotrophic strains were also embedded separately in MMM (controls for back-mutation). The number of colony-forming units (protoplasts and their aggregates) formed during PEG treatment was determined by plating the mixed suspension of protoplasts into MMM supplemented with adenine, methionine, cysteine and uracil.

Nuclear staining. The procedure described by NURSE *et al.* [7] was followed.

Results

Protoplast formation. When only snail enzyme was used, 20% of the cells were converted into protoplasts. When the procedure described by FERENCZY and MARÁZ [8] for *Saccharomyces cerevisiae* was used, the conversion rate was about 30%. The procedure described in the Methods gave 99% protoplasts after 1 h of treatment. Conventionally, protoplasts were regarded as the spherically shaped structures (Fig. 2) which were caused to burst by diluting the osmotically stabilized suspensions with water. Intact *C. utilis* cells differ from these structures by their osmotic stability and ellipsoidal geometry (Fig. 1).

Protoplast fusion. When a mixture of protoplasts prepared from strains VN9-8 and VN15-15 was treated with 35% PEG (MW 6000) in 10 mM CaCl_2 solution and plated in MMM, prototrophic colonies appeared. The fusion frequency based upon nutritional complementation, was calculated as

$$\frac{\text{number of colonies in MMM}}{\text{number of colonies in supplemented MMM}}$$

and gave a value of approximately 1×10^{-3} . When PEG 4000 was used, the fusion frequency was approximately 2×10^{-4} . In the assessment of the fusion frequency it is practical to use as reference base the number of colony-forming units, which is much lower than the number of protoplasts involved in the fusion process. This is due to the fact that a considerable number of protoplasts emerge from anucleate buds and that during PEG treatment an aggregation of protoplasts occurs (Fig. 3).

In a regeneration test with the protoplasts employed in the fusion experiment, only 10% of the protoplasts underwent regeneration and colony formation.

No colonies appeared if intact cells of the two parental strains were cultivated together for mating, or when protoplasts of each partner were

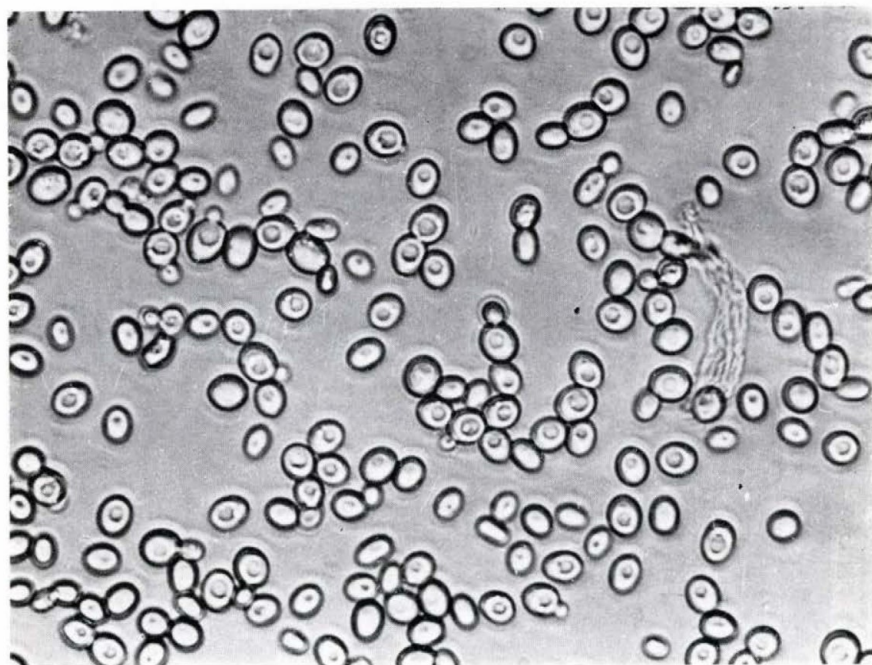


Fig. 1. Cells of *C. utilis* in exponential growth phase. Observation by phase contrast microscopy

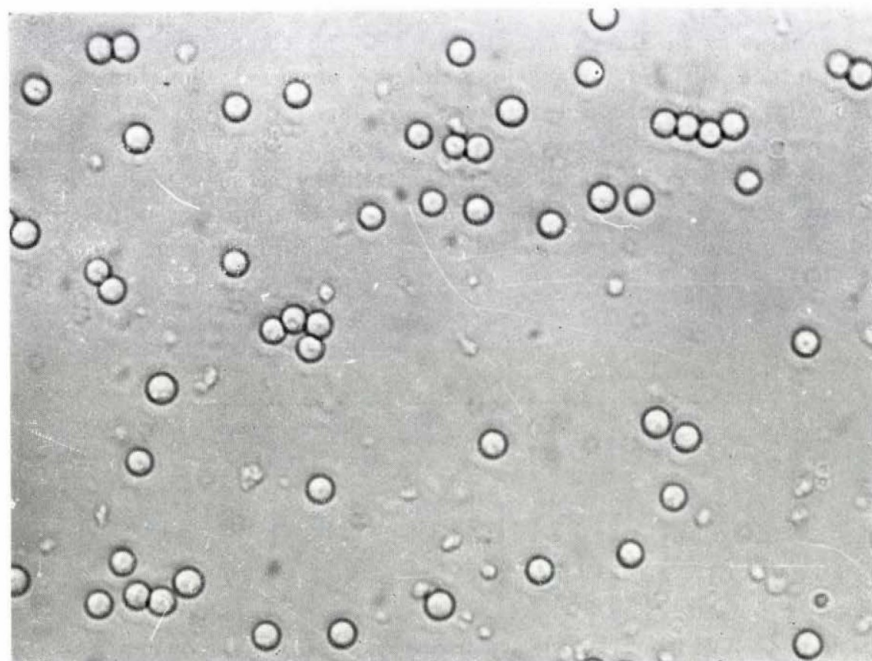


Fig. 2. Protoplasts of *C. utilis* as observed by phase contrast microscopy

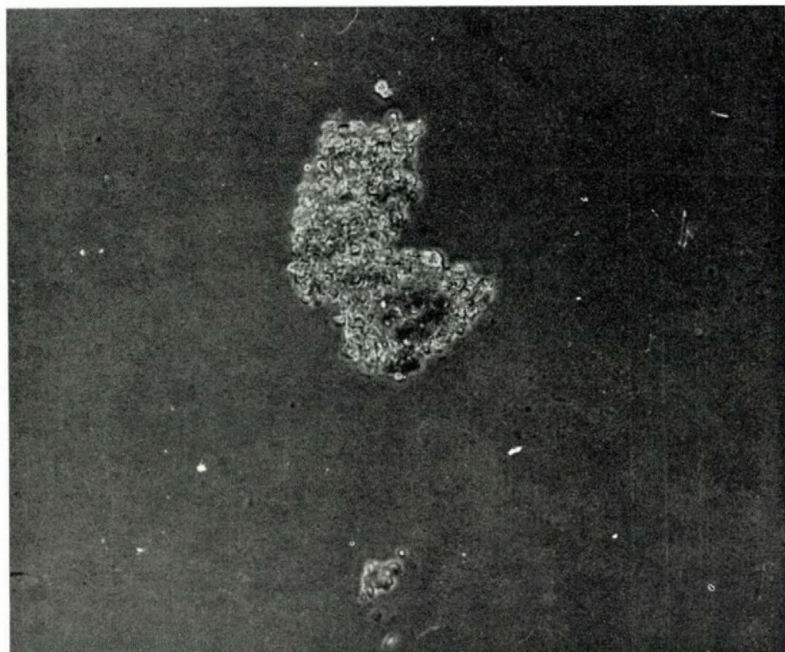


Fig. 3. Protoplasts of *C. utilis* agglutinated under the action of polyethylene glycol

plated separately in MMM. This means that no complementation occurred between intact cells and no back-mutation was observed. Therefore, the prototrophic colonies obtained were fusion products.

Heterokaryon formation. The existence of heterokaryons among the fusion products was indicated by the instability of some fusion products. When prototrophic colonies growing on MMM selection medium were transferred to MM medium, approximately 30% of them failed to produce colonies.

Table I

Segregation of 3 fusion products between strains VN15-15 (*ade₁*, *met₂*) and VM9-8 (*cys₁*, *ura₁*) of *C. utilis*

Hybrid	Total colonies	Prototrophs	Parental types	
			<i>ade₁</i> , <i>met₂</i>	<i>cys₁</i> , <i>ura₁</i>
1	184	20	113	51
2	59	0	59	0
3	152	29	121	2

After 2 days on MM the fusion products were transferred to YPD and the number of auxotrophic cells was analysed by plating a suspension of the cells on YPD and by replica-plating of the resulting colonies onto various supplemented media

This indicates the immediate occurrence of segregation into the parental auxotrophs. In order to elucidate whether the segregants resulted from the dissociation of heterokaryons, the fusion products were allowed to grow for 2 days on MM and then transferred to YPD medium. After 2 days in this medium, a loopful of cells was suspended in sterile water, diluted and spread on solid YPD. The plates were replica-plated on YPD, MM, and MM supplemented with the requirements of both parental strains. Table I shows the results obtained with three fusion isolates.

The high frequency of auxotrophic cells reported in Table I can only be explained if prototrophic cells continue to give rise to auxotrophic ones. This led to the conclusion that the two parental nuclei retained their independence inside the cell.

Cytological evidence of heterokaryons is given in Fig. 4. The number of nuclei per cell was variable (1, 2, 3 or 4 nuclei per cell). This could result from the aggregation and fusion of a number of protoplasts in the presence of PEG (Fig. 3).

Hybrid sporulation. Hybrids which were able to grow in MM medium after several transfers were grown in sporulation medium and after 6 days of incubation at 30 °C, ascus-like formations were apparent in the suspension of prototrophic cells. The degree of sporulation (percentage of cells transformed

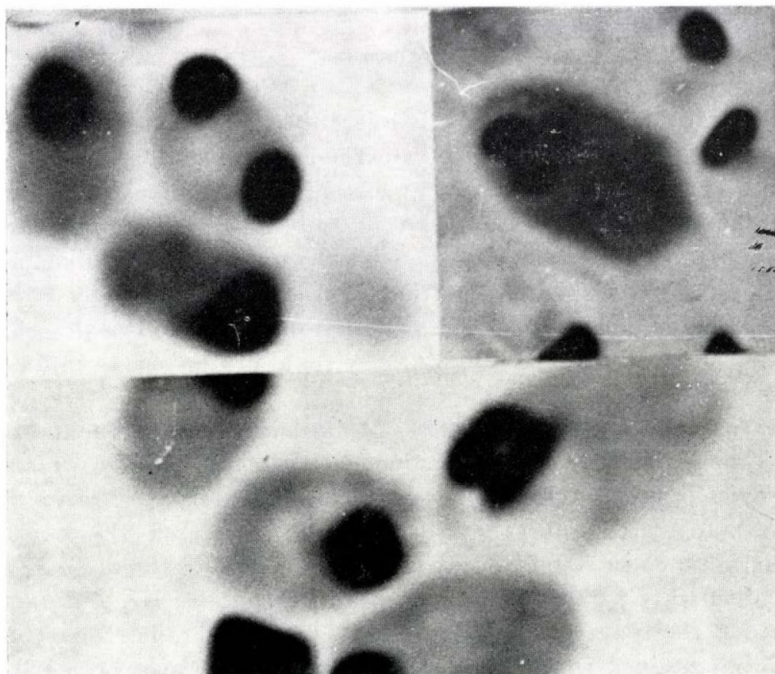


Fig. 4. Nuclear-stained cells of the fusion products

Table II
Proportions of asci with different numbers of spores

Hybrid	Per cent sporulation	Asci (per cent of cells)			
		One-spored	Two-spored	Three-spored	Four-spored
1	90.2	74.2	14.8	0.8	0.0
2	85.4	70.6	12.9	1.0	1.0
3	90.0	77.0	12.0	1.0	0.0
4	85.2	68.7	21.0	2.0	0.3
5	88.0	68.1	18.9	1.0	0.0
6	88.1	73.7	13.7	0.7	0.0
7	86.6	73.8	11.5	1.3	0.0
8	90.2	74.0	11.8	0.4	0.0
9	95.2	79.1	14.8	1.3	0.0
10	82.3	72.2	9.3	0.8	0.0

into asci) was about 90% and the spore diagram (proportions of asci with different numbers of spores) is shown in Table II. The asci contained predominantly one or two spores. Asci with three or four spores were rare.

Discussion

Since EDDY and WILLIAMSON [9] described a procedure to obtain yeast protoplasts with snail enzyme, many methods have been developed [10–12]. These methods include a pretreatment of cells with thiol compounds in order to facilitate the removal of the cell wall and enhance protoplast formation [13, 14]. Our procedure gave higher yields. Extra addition of glucanases and the use of dithiotreitol in the digestion mixture as described by HOUSSET *et al.* [6] were not needed to lyse the cell wall of *C. utilis*. The procedure was further used successfully to obtain a good yield of protoplasts even for stationary-phase cells.

The fusion frequency (1×10^{-3}) obtained in our experiments was very similar to the fusion frequencies obtained for *S. pombe* [15], *A. nidulans* [16] and *C. tropicalis* [17], but higher than that reported for *S. cerevisiae* [18, 19].

The cytological and genetic studies demonstrated the existence of a heterokaryotic phase before stabilization of the diploid stage. Similar results were obtained in *C. tropicalis* [17, 20]. *C. utilis*, however, differs from *C. tropicalis* and the filamentous fungi [21–23] in that the heterokaryotic state cannot be maintained indefinitely. This characteristic may be attributed to the inability of this yeast to form filaments.

Formation of true diploid cells was confirmed by the ability of the fusion isolates to sporulate. The variable number of spores in the ascus-like formations could be due to nuclear division resulting in the failure of certain nuclei to become included in spores [24], or due to the inclusion of two nuclei in certain spores [25].

The protoplast fusion technique has proved a good tool to induce diploid formation and genetic recombination in the yeast *C. utilis*. Further work is in progress to improve the efficiency of the process and to initiate linkage group studies in this yeast.

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MORPHOGENETIC EFFECT OF L-CYSTEINE ON DERMATOPHYTES

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In the presence of L-cysteine, all the 24 dermatophyton fungi under study grew poorly. None of the strains, excepting *Trichophyton mentagrophytes* var. *quinckeanum*, grew in the presence of 0.04 M L-cysteine. The strains growing on a medium containing L-cysteine showed morphological changes. The surface of the colonies lost its velvety appearance and became awnless or waxy. The strains grown in the presence of L-cysteine abundantly formed chlamydospores. The chains of chlamydospores may resemble yeast cell chains, but true budding forms were not found in cultures *in vitro*. If strains precultivated on L-cysteine-containing medium were injected intraperitoneally into mice budding forms appeared in the peritoneal fluid.

The morphogenetic effect of the environment on fungi is well known [1, 2]. The phenomenon has been utilized in the identification of species of some groups of fungi, using selective media [3–7]. One of the most commonly used additives of morphogenetic effect is L-cysteine, a reductant due to its free SH-groups [8–10]. The morphogenetic effects of the host on parasitic (pathogenic) fungi are also well known [11, 12]. Both of these factors support fungal species existing in hyphal (mycelial) form in their saprobic life to turn into existence in yeast form (M → Y transformation) [13–17].

Of the morphogenetic phenomena occurring in dermatophytes, mainly the reversible ($C \rightleftharpoons M$) and irreversible ($C \rightarrow M$) loss of conidiogenesis has been studied.

In the present investigations, an attempt was made to widen or generalize the results published by RIPPON and SCHERR [18]. These authors described the M → Y transformation of the fungi *Microsporum audouinii* and *Trichophyton rubrum* on a medium containing L-cysteine. Moreover they found yeast cells when cystein-grown strains had been implanted into mice.

Materials and methods

Fungi. Twenty four strains, belonging to 19 fungal species, were used in the experiments. They were, *Epidermophyton floccosum* §V, *Microsporum boullardii* §10, *M. cookei* §4, *M. felineum* §12, *M. gypseum* §6, *M. nanum* §3, *M. persicolor* §9, *M. racemosum* §11, *M. vanbreuseghemii* §8, *Trichophyton ajelloi* §11, *T. gloriae* §20, *T. longifusum* §1, *T. mentagrophytes* var. *erinacei* §13, *T. mentagrophytes* var. *mentagrophytes* §28, §29, §30, *T. mentagrophytes* var. *quinckeanum* §19, *T. phaseoliforme* §22, *T. rubrum* §24, *T. simii* §21, *T. terrestre* §16a, §16b, *T. vanbreuseghemii* §38 and *T. violaceum* §18, isolated from clinical material or soil samples and maintained in our strain collection.

Medium. SWARTZ and GEORG's agar [19] of the composition: Casamino acid, 5.0 g; glucose, 40.0 g; $\text{MgSO}_4 \cdot 7 \text{H}_2\text{O}$, 0.15 g; trace element solution, 0.5 ml; Sørensen's phosphate buffer (1/15 M, pH 6.8) 200 ml; agar-agar, 15.0 g; distilled water to 1000 ml.

The trace element solution consisted of HBO_3 , 28.5 mg; $\text{CuSO}_4 \cdot 5 \text{H}_2\text{O}$, 93.0 mg; MoO_3 (85%), 17.6 mg; $\text{Fe}_2\text{SO}_4 \cdot (\text{NH}_4)_2\text{SO}_4 \cdot 24 \text{H}_2\text{O}$, 865.0 mg; MnSO_4 , 30.5 mg; $\text{ZnSO}_4 \cdot 7 \text{H}_2\text{O}$, 395 mg in distilled water to 500 ml.

The completed medium was sterilized at 120 °C for 10 min. The control medium contained no cysteine. In the media used in the experiments, the L-cysteine concentrations were 0.1, 0.08, 0.06, 0.04, 0.02 and 0.01 M. The L-cysteine solutions were sterilized by filtering through Millipore membranes (pore diameter 0.45 μm).

The autoclaved medium was cooled to 50 °C, then the corresponding L-cysteine solution was mixed to it and 20–25 ml medium was poured into Petri dishes. The inoculated media were incubated at 37 °C for 14 days. Micromorphology was examined after embedding in a lactophenol fixative. If it was necessary, the Congo-red intensified polarization process was applied to study cell wall morphology.

Table I

Growth and "chlamydospore" formation of dermatophytes on Swartz's medium with or without L-cysteine

Dermatophyte species or strain	Growth and chlamydospore formation			Inhibition per cent*
	without cysteine	with 0.01 M	with 0.02 M	
		cysteine		
<i>E. floccosum</i>	+ chl	+ CHL	—	55
<i>M. boullardii</i>	+ chl	+ CHL	+ CHL	23
<i>M. cookei</i>	(+)	(+) CHL	—	50
<i>M. felineum</i>	+ chl	+ CHL	—	53
<i>M. gypseum</i>	+	+ CHL	+ CHL	33
<i>M. nanum</i>	+	+ chl	—	47
<i>M. persicolor</i>	+ chl	+ CHL	+ CHL	33
<i>M. racemosum</i>	(+)	—	—	100
<i>M. vanbreuseghemii</i>	+	+ chl	+ CHL	30
<i>T. ajelloi</i>	(+)	(+)	—	37
<i>T. glorieae</i>	(+) chl	+ CHL	—	40
<i>T. longifusum</i>	+	+ CHL	—	41
<i>T. mentagrophytes</i> var. <i>erinacei</i>	+	+ chl	+ CHL	29
<i>T. mentagrophytes</i> var. <i>mentagrophytes</i> §28	+	+ chl	—	40
<i>T. mentagrophytes</i> var. <i>mentagrophytes</i> §29	+	+ chl	—	43
<i>T. mentagrophytes</i> var. <i>mentagrophytes</i> §30	+	+ chl	+ CHL	31
<i>T. mentagrophytes</i> var. <i>quinckeanum</i> !	+ chl	+ CHL	+ CHL	20
<i>T. phaseoliforme</i>	+ chl	+ CHL	—	40
<i>T. rubrum</i>	+ chl	+ CHL	—	46
<i>T. simii</i>	+	—	—	100
<i>T. terrestre</i> §16a	(+)	—	—	100
<i>T. terrestre</i> §16b	+	+ chl	—	37
<i>T. vanbreuseghemii</i>	+	+ chl	—	55
<i>T. violaceum</i>	(+) chl	—	—	100

— = no growth, (+) = weak growth, + = good growth, chl = weak chlamydospore formation, CHL = strong chlamydospore formation, ! = growth in the presence of 0.04 M L-cysteine.

* Calculated for the growth in the presence of 0.01 M L-cysteine.

The species showing well-defined morphological changes while growing in L-cysteine-containing medium were further examined in albino mice of about 25 g body weight. Colonies grown on medium containing 0.02 M (in the case of *E. floccosum* and *M. gypseum*, 0.01 M) L-cysteine were suspended in 0.85% saline, using a Potter homogenizer. Of these suspensions, mice were injected i.p. with 0.5 ml each. Mice were killed on days 5, 10, 15 and 20 postinoculation. The peritoneal fluid was examined in periodic acid-Schiff stained preparations, and attempts were made to re-isolate the strains on Sabouraud glucose agar containing chloramphenicol and actidione.

Results

In Swartz's medium containing no cysteine, the strains *T. ajelloi*, *T. terrestre* §16a, *T. glorieae*, *T. violaceum*, *M. cookei* and *M. racemosum* grew less abundantly than the others. The velvety appearance of the colonies remained

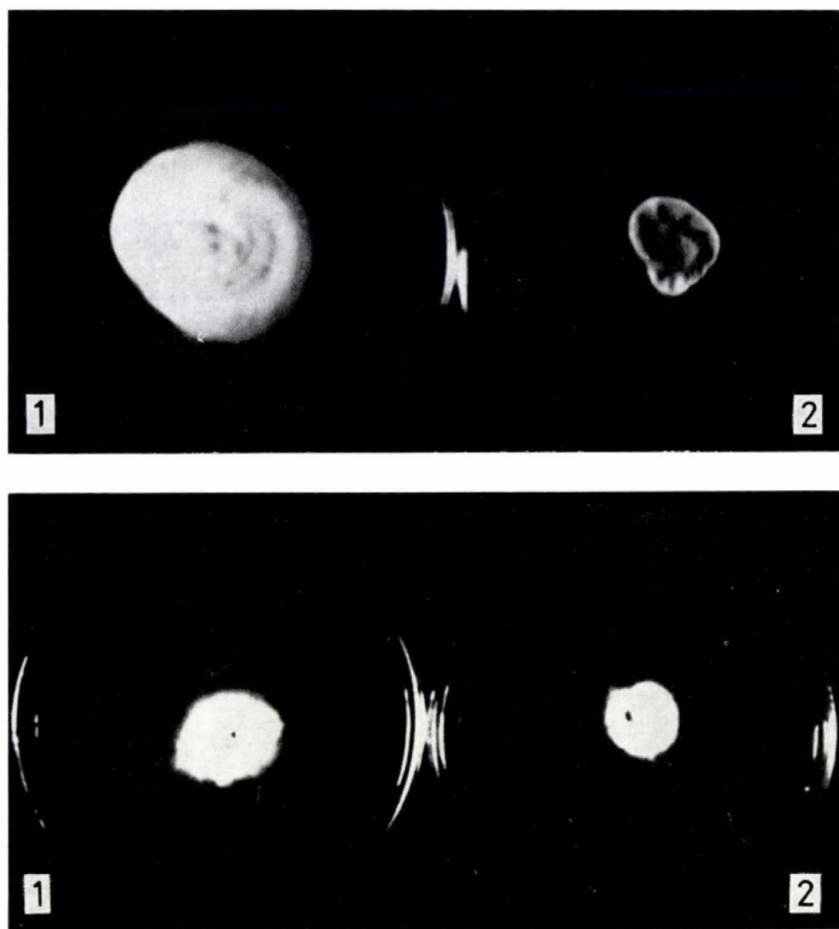


Plate I. Upper photos. 1. A colony of *T. vanbreuseghemii* on control medium containing no cysteine; 2. A colony of *T. vanbreuseghemii* on the medium containing 0.01 M L-cysteine. Lower photos. 3. A colony of *T. mentagrophytes* var. *quinckeanum* on control medium; 4. A colony of *T. mentagrophytes* var. *quinckeanum* on medium containing 0.02 M L-cysteine



Plate II. 1. Filaments of *T. mentagrophytes* var. *quinckeanum* grown on control medium. Lactophenol preparation, $\times 620$. 2. Chlamydospores of *T. mentagrophytes* var. *quinckeanum* grown on medium containing 0.02 M L-cysteine. Lactophenol preparation $\times 700$. 3. Filaments and a few chlamydospores of *E. floccosum* from colonies grown on control medium. Lactophenol preparation, $\times 680$. 4. Chlamydospores of *E. floccosum* from colonies grown on medium containing 0.01 M L-cysteine. Lactophenol preparation, $\times 680$

unchanged, but the colonies of *T. rubrum* and *M. gypseum* appeared less velvety. The colonies were white, their reverse side was yellow or yellowish-brown. The reverse side of *T. rubrum* colonies was lilac, indicating that the diagnostic marker of *T. rubrum* remained constant. Most of the strains formed microconidia abundantly, but only a few species produced large amounts of macroconidia and chlamydospores.

On medium containing 0.01 M L-cysteine, the 4 strains *T. terrestre* §16a, *T. simii*, *T. violaceum* and *M. racemosum* did not grow at all, the others grew slowly (Table I, and Plates I and II).

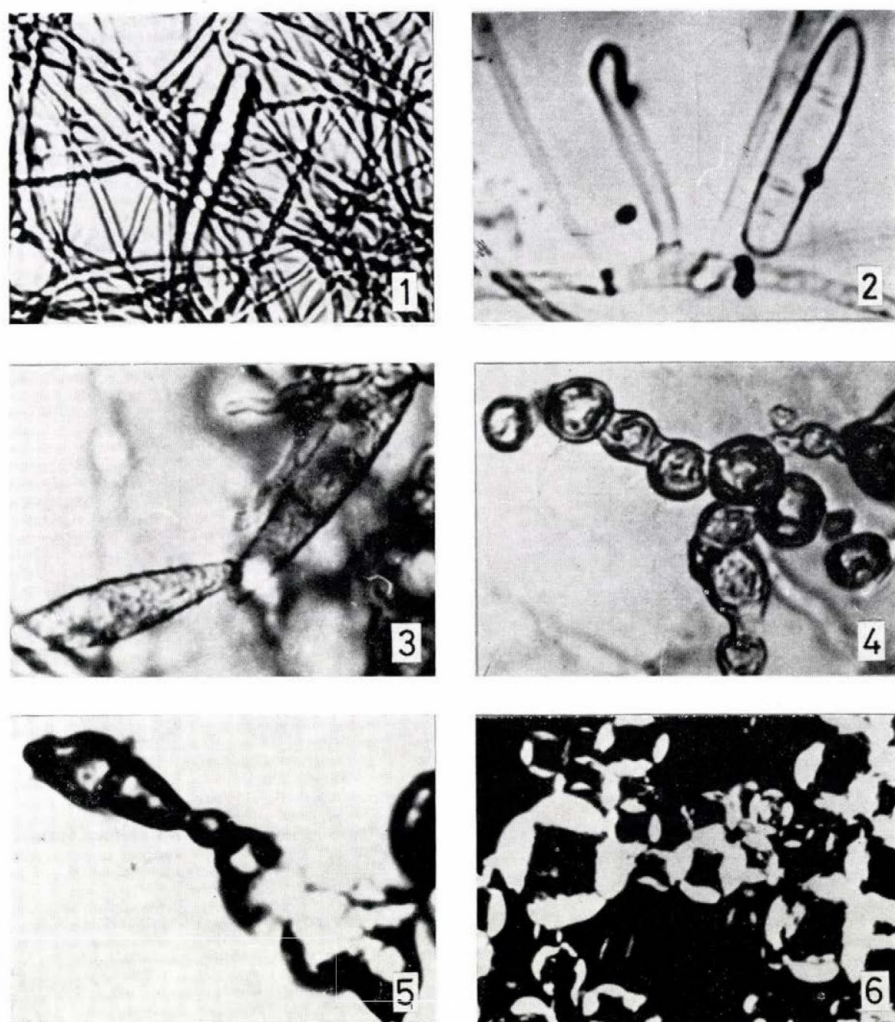


Plate III. 1. A young macroconidium and filaments of *M. gypseum* grown on control medium. Lactophenol preparation, $\times 550$. 2. A mature macroconidium and filaments from a colony of *M. gypseum* grown on control medium. Lactophenol preparation, $\times 1200$. 3. Macroconidia and chlamydospores from a colony of *M. gypseum* grown on medium containing 0.02 M L-cysteine. Lactophenol preparation, $\times 700$. 4. Chlamydospore chains of *M. gypseum* grown on medium containing 0.02 M L-cysteine. Lactophenol preparation, $\times 650$. 5. A macroconidium of *M. gypseum* on a conidiophore transformed into chlamydospores. The colonies were grown on medium containing 0.02 M L-cysteine. Lactophenol preparation, $\times 680$. 6. Chlamydospores of *M. gypseum* grown on medium containing 0.02 M L-cysteine. Polarization microscopy, intensified with Congo red. $\times 350$

Table II

Macro- and microconidium formation by dermatophytes in Swartz's medium containing no cysteine or 0.01 M L-cysteine

Dermatophyton species or strain	Microconidium	Macroconidium	Microconidium	Macroconidium
	in the absence of L-cysteine		in the presence of L-cysteine	
<i>E. floccosum</i>	—	+	—	+
<i>M. boullardii</i>	—	+	—	(+)
<i>M. cookei</i>	+	—	+	—
<i>M. felineum</i>	+	—	(+)	—
<i>M. gypseum</i>	+	+	+	+
<i>M. nanum</i>	+	+	+	(+)
<i>M. persicolor</i>	+	—	+	—
<i>M. racemosum</i>	+	—	/	/
<i>M. vanbreuseghemii</i>	+	—	+	—
<i>T. ajelloi</i>	—	++	—	++
<i>T. glorieae</i>	+	—	+	—
<i>T. longifusum</i>	—	+	—	(+)
<i>T. mentagrophytes</i> var. <i>erinacei</i>	+	—	+	—
<i>T. mentagrophytes</i> var. <i>mentagrophytes</i> §28	+	—	+	—
<i>T. mentagrophytes</i> var. <i>mentagrophytes</i> §29	+	+	+	(+)
<i>T. mentagrophytes</i> var. <i>mentagrophytes</i> §30	+	—	+	—
<i>T. mentagrophytes</i> var. <i>quinckeanum</i>	+	—	+	—
<i>T. phaseoliforme</i>	+	—	+	—
<i>T. rubrum</i>	+	—	+	—
<i>T. simii</i>	+	—	/	/
<i>T. terrestre</i> §16a	++	—	/	/
<i>T. terrestre</i> §16b	+	—	+	—
<i>T. vanbreuseghemii</i>	+	+	+	(+)
<i>T. violaceum</i>	—	—	/	/

++ = many, + = few, (+) = very few, — = no conidium; / = no colony formation.

On medium containing 0.02 M L-cysteine only one strain of each of 5 species and 2 varieties showed growth (Table I and Plates II and III). Even these, viz. *T. mentagrophytes* var. *mentagrophytes* §30, *T. mentagrophytes* var. *erinacei*, *T. mentagrophytes* var. *quinckeanum*, *M. boullardii*, *M. persicolor*, *M. vanbreuseghemii* and *M. gypseum* grew slowly.

In the presence of 0.04 M L-cysteine, *T. mentagrophytes* var. *quinckeanum* was the only strain showing growth. Even this strain grew slowly (Table I).

No growth was observed in media containing 0.06–0.1 M L-cysteine.

The surface of the dermatophyte colonies grown on Swartz's medium containing L-cysteine was generally less compact and less velvety than that of the control colonies. Some species (strains) e.g. *T. vanbreuseghemii*, *T. longi-*

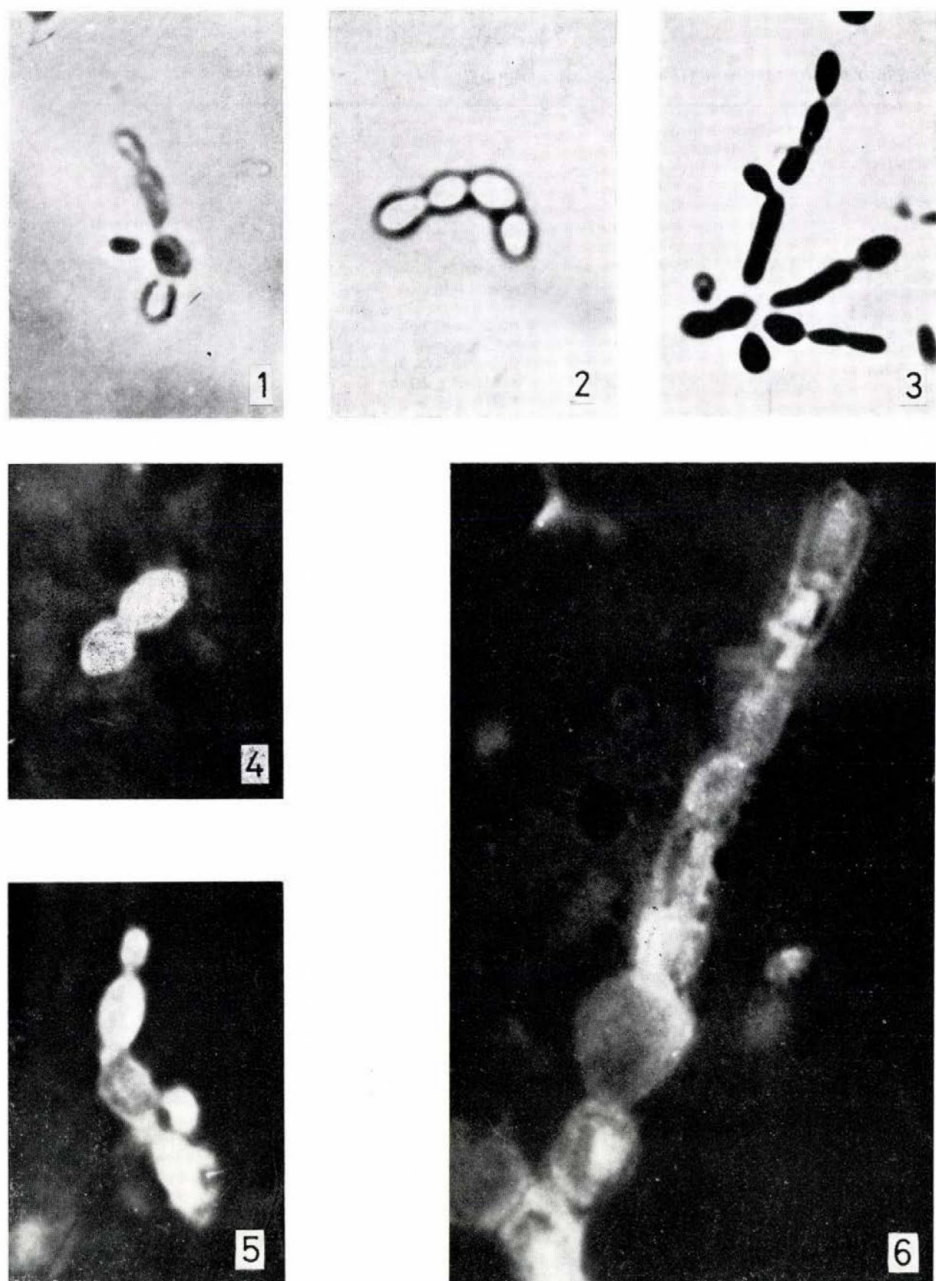


Plate IV. 1, 2 and 3. Budding form of *T. mentagrophytes* var. *mentagrophytes* §30 obtained from the peritoneal fluid of a mouse inoculated 10 days earlier. Water-glycerol preparations, $\times 1200$, $\times 1100$ and $\times 1300$. 4 and 5. Budding forms of *T. mentagrophytes* var. *mentagrophytes* §30 from the peritoneal fluid of a mouse inoculated 10 days earlier. Schiff stain. Negative copy from a positive colour photo, $\times 1500$. 6. Budding form of *M. gypseum* from the peritoneal fluid of a mouse inoculated 10 days earlier. Schiff stain. Negative copy from a positive colour photo, $\times 2600$.

Table III

Behaviour in the mouse peritoneum of dermatophytes precultivated in L-cysteine-containing medium

Dermatophyte species (strain)	Examination for	Days after inoculation			
		5	10	15	20
<i>E. floccosum</i>	budding form	+	+	—	—
	reisolation	—	—	—	—
<i>M. gypseum</i>	budding form	+	+	+	—
	reisolation	+	+	+	—
<i>M. vanbreuseghemii</i>	budding form	+	+	—	—
	reisolation	—	—	—	—
<i>T. mentagrophytes</i> var. <i>erinacei</i>	budding form	+	+	—	—
	reisolation	+	+	—	—
<i>T. mentagrophytes</i> var. <i>mentagrophytes</i> §30	budding form	+	+	—	—
	reisolation	+	+	—	—
<i>T. mentagrophytes</i> var. <i>quinckeanum</i>	budding form	+	+	+	+
	reisolation	+	+	+	+

fusum, *E. floccosum*, developed winding colonies, sometimes with awnless or waxy surface. The colonies were dirty white, with brownish-yellow, in the case of *T. rubrum* dark lilac, reverse side.

The formation of micro- and macroconidia (by species or strains capable of forming these at all) was often weak (Table II) in the presence of L-cysteine.

An intensive production of chlamydospore-like formations was observed on cysteine-containing media in the colonies of all the species able to grow on such media (Table I, Plates I and II). The chlamydospores were heterogeneous in shape and size, their wall was uneven in thickness. Deformations of mycelia were also observed.

In smears prepared from peritoneal fluid, the inoculated fungus was demonstrable and also showed yeast-like cells at least up to the 10th day of incubation (Plates III and IV). All the strains re-inoculated into Sabouraud agar, except the two species *E. floccosum* and *M. vanbreuseghemii* that could not be recultivated, showed their usual morphological markers.

Discussion

Cultivation of dermatophyton fungi at 37 °C on Swartz's medium containing L-cysteine resulted in major morphological changes. The colonies grew at a lower rate, they tended to be less compact, and awnless and waxy in surface. These phenomena were enhanced and the growth of the strains was increasingly inhibited if the L-cysteine concentration was raised. E.g., in the presence of 0.04 M L-cysteine, *T. mentagrophytes*, var. *quinckeanum* was

the only strain growing, and none of the strains could be cultivated in media containing 0.06 M or more L-cysteine.

The growth-inhibiting activity of L-cysteine has already been observed by other authors in cultures of the dermatophyton fungi *T. rubrum*, *T. mentagrophytes*, *T. concentricum*, *M. canis* and *M. gypseum* [20–25], of other moulds, viz. *Helminthosporium carboneum* and *Aspergillus niger* [26, 27], and of bacteria viz. *Peptostreptococcus anaerobius* and *Escherichia coli* [28, 29].

We have failed to reproduce the micromorphological changes reported by RIPPON and SCHERR [18]. Our attempts to obtain budding cells or chains reminding of chains of yeast cells on L-cysteine media have also failed. Instead, chlamydospore-like formations appeared in the colony layer adjacent to the medium surface, or such formations increased in number, as compared to the control colonies. These chlamydospore-like forms were examined in detail.

We have found that (i) the chlamydospore-like formations are directly related in number to the L-cysteine concentration in the medium; (ii) they never show budding; (iii) their wall is irregularly thickened. This third phenomenon was surprising, for the chlamydospore-like structures became distorted on pressing of the coverslip. The thickening, in spite of its wavy irregular shape, covers a considerably arranged structure showing a well-defined anisotropy, i.e. birefringence in preparations stained with 1% Congo red. In direct polarization, however, this birefringence was very weak. It should be noted that, e.g., the evenly thick wall of the microconidia of *M. gypseum* shows no anisotropy in directly examined preparations and a very weak one on Congo red intensification.

It seems therefore reasonable (i) to regard the above-described structures as chlamydospores and (ii) to assume that the enhanced chlamydospore production represents the primary morphological effect of L-cysteine.

In the peritoneal exudate of mice, we observed M → Y transformation of strains (species) precultivated on L-cysteine-containing medium, a medium unusual in the cultivation of dermatophytes. Formations consisting of isolated yeast cells as well as pseudomycelium-like and pseudohypha-like structures (catenate yeast cells) developed instead of the usual filamentous forms. These observations have supported, and extended to further species, the original observations of RIPPON and SCHERR [18] on the correlation between the penetration of infection on the one hand and the morphogenesis on the other. In this respect, three facts should be taken into account.

First, slow growth, compact structures and waxy surface on Sabouraud agar are characteristic of the dermatophyton fungi (*T. verrucosum*, *T. violaceum* and *T. schoenleini*) causing invasive and persisting infections. These properties resemble those acquired by other species while growing in the presence of L-cysteine. Second, the morphogenetical changes observed *in vivo* should not be overestimated. The results reported by RIPPON and SCHERR

[18], in accordance with our results, indicate that the dermatophytes introduced into the depth of systemic infections, i.e. beyond the cutaneous barrier, tend to be similar in shape to the tissue invading forms of dimorphous fungi spontaneously pathogenic in deep tissues. This transformation allows survival in the host organism, but does not indicate pathogenicity. Third, some recent publications suggest that dermatophytes may attack tissues other than the cutis. From the affected organs, the fungus can be isolated as the exclusive pathogen and the pathological processes respond well to specific therapy. From such processes *T. violaceum*, *T. verrucosum* and *Achorion schoenleini* have been isolated most frequently. In the few histological preparations examined from the attacked tissues [30–37] structures designated as chlamydospores or arthrospores were found. Nevertheless, the appearance of these structures reminds us, first of all, of yeast cells.

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FORMALIN STRESS REACTION OF GERMFREE AND CONVENTIONAL MICE TREATED WITH *BORDETELLA PERTUSSIS* VACCINE

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Germfree and conventional mice responded similarly to pertussis vaccine treatment. In both groups, lymphocytosis and splenomegaly developed in a similar proportion. The formalin stress reaction of germfree and conventional mice with hypertrophic lymphoid organs induced by pertussis vaccine differed from that of untreated mice: the treated germfree and conventional mice showed an acute increase of lymphocytosis without any significant change in splenomegaly.

Since SELYE's reports [1–3] it is well known that a healthy organism subjected to various stressors, reacts with a regularly developing physiological reaction, the General Adaptational Syndrome. Its first stage, the alarm reaction, is characterized by lymphopenia appearing in 6 h following the stress. In this case the number of circulating lymphocytes decreases by 50% and, at 18 to 24 h following the stress the lymphoid organs decrease considerably in weight.

It is also known that *Bordetella pertussis* vaccine causes an increase in the total white blood cell count, due mostly to lymphocytosis, and splenomegaly too develops [4, 5]. The general adaptational reactions of pertussis vaccine treated mice with hypertrophic lymphoid organs take place in different forms, and increased cold sensitivity was found in the response to cold stress [6].

Germfree mice have under-developed lymphoid systems. The functional state of their immune systems remains on the newborn level owing to the scarcity of antigenic stimuli in the environment. At the same time, there is a change in their alarm reaction to cold stress: they exhibit an increased susceptibility to cold [7].

Pertussis vaccine affects the underdeveloped lymphoid systems of germ-free mice as well; an increase in total white blood cell count and splenomegaly have been described [8]. The effect of pertussis vaccine on the state of the lymphoid system is most expressed on the 4th to 8th days following treatment [9].

The present study deals with the response to formalin stress of germfree and conventional mice with a hypertrophic lymphoid system due to previous pertussis vaccine treatment. Formalin stress was applied on the 5th day following vaccine treatment.

Materials and methods

Experimental animals. Germfree and conventional, four to five-week-old C3Hmg mice of both sexes were used (LATI, Gödöllő, Hungary).

Germfree state was maintained with plastic isolators (VELAZ P-01, Czechoslovakia). The germfree mice were fed with gamma ray sterilized pellets and autoclaved water ad libitum. The germfree state of the outer and inner environment of the mice during the experiment was controlled by culture media accepted in the literature [10].

Bordetella pertussis vaccine. The vaccine contained 30×10^9 /ml killed bacterium suspended in physiological saline (Institute for Serobacteriological Production and Research Human, Budapest). The mice received single intraperitoneal injections of 0.3 ml vaccine containing 9×10^9 bacteria. The control mice were treated with physiological saline administered in the same way and quantity.

Formalin stress. A single dose of 0.1 ml 4% formalin solution was administered subcutaneously. The control mice received physiological saline in the same way and quantity.

Absolute lymphocyte count and lymphocyte index were determined from blood taken from the caudal vein under standardized conditions.

$$\text{Lymphocyte index} = \frac{\text{mean absolute lymphocyte count in the treated group}}{\text{mean absolute lymphocyte count in the control group}}$$

Relative spleen weight and spleen index of the sacrificed animals were determined as follows.

$$\text{Relative spleen weight} = \frac{\text{spleen weight mg}}{\text{body weight g}}$$

$$\text{Spleen index} = \frac{\text{mean relative spleen weight in the treated group}}{\text{mean relative spleen weight in the control group}}$$

Statistical evaluation of data. Evaluation of the results was carried out by Student's two-sample *t* test. The accepted level of significance was $p = 0.05$.

Results

In the present experiment 28 germfree (GF) and 28 conventional (CV) mice were treated with pertussis vaccine, and simultaneously, 28 GF and 28 CV mice received physiological saline. A group of both the vaccinated and the physiological saline treated mice was subjected to formalin stress on the 5th day following treatment. The rest of the mice received physiological saline. Table I demonstrates the experimental groups, their numbers and the treatments.

Absolute lymphocyte count determination was performed immediately before eliciting the formalin stress (zero hour) then in the 6th and 24th h. Relative spleen weight determination was carried out in the different experi-

Table I
Mouse groups and treatments

Mice	Group	No. of mice	Treatments	
			intraperitoneal	subcutaneous
Germfree	GF-P-F	16	pertussis vaccine	formalin
	GF-P	12	pertussis vaccine	physiological saline
	GF-F	16	physiological saline	formalin
	GF	12	physiological saline	physiological saline
Conventional	CV-P-F	16	pertussis vaccine	formalin
	CV-P	12	pertussis vaccine	physiological saline
	CV-F	16	physiological saline	formalin
	CV	12	physiological saline	physiological saline

Table II

Mean absolute lymphocyte count and mean relative spleen weight of pertussis vaccine-treated and control germfree and conventional mice (zero hour)

Group	Mean absolute lymphocyte count, $\times 10^{12}/\text{litre}$	Mean relative spleen weight
GF	3.0 ± 0.42	2.6 ± 0.6
CV	4.3 ± 0.50	4.4 ± 0.6
GF-P	9.27 ± 0.45	6.9 ± 2.0
CV-P	13.0 ± 1.40	10.6 ± 3.6

Significance

	Absolute lymphocyte count	Relative spleen weight
FG versus CV	$p < 0.001$	$p < 0.001$
GF versus GF-P	$p < 0.001$	$p < 0.001$
CV versus CV-P	$p < 0.001$	$p < 0.001$
GF-P versus CV-P	$p < 0.001$	$p > 0.05$

mental groups by sacrificing 6 mice at zero min and again at 24 h. The experiment was stopped at 24 h after elicitation of the stress.

Mean absolute lymphocyte count and mean relative spleen weight in the GF, CV, GF-P and CV-P groups, determined at 0 h are summarized in Table II.

The data in Table II show that the absolute lymphocyte count and relative spleen weight of GF mice were significantly lower than the corresponding values obtained in the CV group. On the other hand, a significantly higher absolute lymphocyte count and relative spleen weight were obtained in the groups treated with pertussis vaccine (GF-P, CV-P). This means that a signif-

icant lymphocytosis and an increase in relative spleen weight occurred in both GF and CV mice on the 5th day following vaccine treatment.

Mean absolute lymphocyte count and mean relative spleen weight determined at 24 h in the groups indicated in Table II did not differ from the values obtained at 0 h.

The changes due to formalin stress in the absolute lymphocyte count are indicated for each group in Fig. 1.

In the CV-F group the mean absolute lymphocyte count in the 6th h of the stress reaction decreased to one third of the value obtained before the stress reaction. The values obtained in the 24th h showed the lymphopenia to disappear. On the other hand, in the 6th h no change in the absolute lymphocyte count was observed in the GF-F group, while the values obtained in the 24th h were significantly lower than at 0 h.

The GF and CV mice responding with lymphocytosis to pertussis vaccine (groups GF-P-F and CV-P-F) showed a significant increase in lymphocyte count in the 6th h of the stress reaction as compared to the values obtained at 0 h. In the 24th h, however, the absolute lymphocyte count did not differ from that obtained at 0 h.

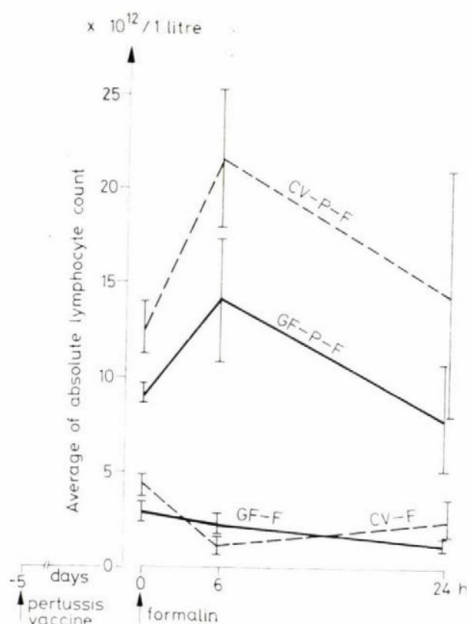


Fig. 1. Changes due to formalin stress in absolute lymphocyte count in pertussis vaccine-treated and control germfree and conventional groups. Significance: GF-F (0 h) versus GF-F (6th h) $p > 0.05$; GF-F (0 h) versus GF-F (24th h) $p < 0.001$; GF-P-F (0 h) versus GF-P-F (6th h) $p < 0.001$; GF-P-F (0 h) versus GF-P-F (24th h) $p > 0.05$; CV-F (0 h) versus CV-F (6th h) $p < 0.001$; CV-F (0 h) versus CV-F (24th h) $p < 0.001$; CV-P-F (0 h) versus CV-P-F (6th h) $p < 0.001$; CV-P-F (0 h) versus CV-P-F (24th h) $p > 0.05$; CV-P-F (0 h) versus GF-P-F (6th h) $p > 0.05$.

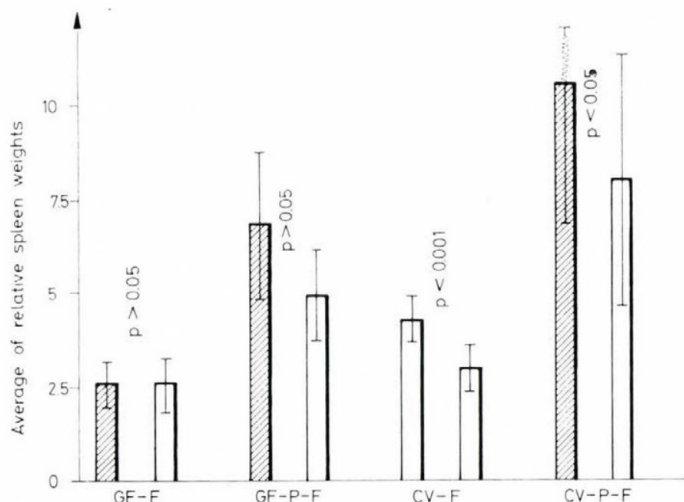


Fig. 2. Mean relative spleen weight determined before and after formalin stress in pertussis vaccine-treated and control germfree and conventional mice. Shaded columns, 0 h; open columns, 24th h

Figure 2 shows the mean relative spleen weight determined immediately before the formalin stress reaction and again in the 24th h.

In the CV-F group the mean relative spleen weight was significantly lower in the 24th h than at 0 h with no significant change of the parameter in the other groups.

Discussion

In accordance with the literature, the present experiments also indicated the splenomegaly inducing effect of *B. pertussis* vaccine in germfree mice [8] and, in addition, an expressed lymphocytosis in their peripheral blood, similar to that observed in conventional mice. The mean absolute lymphocyte count in the vaccine treated germfree group (GF-P) was below that in the vaccine treated conventional mice (CV-P) — cp. Table II — GP-P versus CV-P: $p < 0.001$. The increase in absolute lymphocyte count and in relative spleen weight was approximately identical in the GF-P and the CV-P groups as demonstrated by the index values for lymphocytes and spleen weights (Table III).

Thus, germfree mice with an underdeveloped lymphoid system responded to pertussis vaccine like conventional mice of the same age with a normally developed immune system in respect of absolute lymphocyte count and relative spleen weight.

In agreement with our previous results [9], the response to stress of GF mice was different from the response of CV mice, since the lymphopenic reac-

Table III*Index values for lymphocytes and spleen weight in GF-P and CV-P group (zero hour)*

Group	Lymphocyte index	Spleen index
GF-P	3.2	2.4
CV-P	3.1	2.6

tion and splenic atrophy in conventional mice (CV-F) regularly occurring after stress did not appear in germfree mice (GF-F).

A regular lymphopenic reaction and splenic atrophy following formalin stress failed to evolve in either group treated with pertussis vaccine (CV-P-F, GF-P-F) but in the 6th h both showed an acute increase in the previously developed lymphocytosis which disappeared then by the 24th h. Although, in the 6th h the absolute lymphocyte count in the GF-P-F group was significantly lower than in the CV-P-F group, the rate of increase, measured by the 0 h values, was essentially identical in both groups (lymphocyte indices: 1.6 and 1.7, respectively).

In vaccine treated GF mice the absolute lymphocyte count in the 6th h after formalin stress (GF-P-F group) corresponded well to the value obtained in vaccine treated conventional mice before the stress reaction (0 h). The vaccine treated germfree and conventional mice responded to stress similarly in regard of mean spleen weight, i.e., there was no significant loss in relative spleen weight in these groups (GF-P-F and CV-P-F) in the 24th h as compared to 0 h.

Thus, the pertussis vaccine changed the response to formalin stress of the lymphoid systems of both CV and GF mice in the same direction; both GF and CV pertussis vaccine treated mice reacted to formalin stress with an acute increase in lymphocyte count and no significant change in spleen weight.

The results, thus, showed no difference in the non-specific lymphoid response of pertussis vaccine treated germfree and conventional mice. The vaccine caused lymphocytosis and splenomegaly in both conventional and germfree mice, and both germfree and conventional mice with lymphocytosis and splenomegaly elicited by the vaccine, responded similarly to stress.

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ORIGIN AND SPREAD OF *PSEUDOMONAS AERUGINOSA*, *PROTEUS* AND *KLEBSIELLA* DURING TWENTY YEARS IN AN INFECTIOUS HOSPITAL

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Bacteriological examinations carried out in an infectious hospital revealed that the occurrence of *Pseudomonas* and *Proteus* grew 4-fold, and the rate of *Klebsiella* positive cultures 3.5-fold between 1958 and 1977. On the other hand, the occurrence of *Staphylococcus aureus* decreased to the half since 1961. The occurrence of Gram-negative facultative pathogens started to increase in the surgical wards in the fifties and the rise lasted until the mid-sixties. *Pseudomonas aeruginosa* was the most frequent among them. In contrast, *Escherichia coli* dominated and *P. aeruginosa* was the least frequent in the non-surgical wards. Here the Gram-negative facultative pathogens showed a more rapid increase and the incidence of *P. aeruginosa* and *Proteus* kept rising throughout the whole examination period. When Gram-negative facultative pathogens of hospital origin were colonizing, the proportion of sick persons versus symptomless carriers was significantly higher than in the case of extrahospital colonization on the basis of the records of 300 *P. aeruginosa*, 300 *Proteus* and 300 *Klebsiella* positive patients. This proportion changed parallel with the rate of the strains of hospital origin. The number of patients who acquired *P. aeruginosa*, *Proteus* or *Klebsiella* extrahospitally, kept continuously rising between 1958 and 1971. Thus, the advance of Gram-negative facultative pathogens is due not only to nosocomial causes.

Since the late fifties the occurrence of nosocomial infections due to *Staphylococcus aureus* started to decrease all over the world and simultaneously Gram-negative facultative pathogens (GNFP) became the main hospital infective agents [1–8]. Owing to this, the incidence of lethal cases grew as well, as the lethality of infections caused by *Pseudomonas aeruginosa*, *Proteus* and *Klebsiella* surpassed that of other pathogens [8, 9]. Several papers have called attention to the danger of the changed conditions [10–12].

The question arose when and to what extent this change took place in Hungary, which was the dynamism of the spread of GNFP, and whether their occurrence increased only in hospitals or factors of their spread could be found also outside hospitals. To answer these questions the changes occurring in the number of *P. aeruginosa*, *Proteus* and *Klebsiella* positive cultures deriving from non-faecal pathological materials in the László Central Hospital for Infectious Diseases and the case records of 900 GNFP positive patients hospitalized between 1958 and 1977 were studied.

Escherichia coli findings have been taken into consideration but not analysed, as *E. coli* forms a significant part of the normal intestinal flora and thus its epidemiology differs from that of *Pseudomonas* or *Klebsiella*. Finally, *S. aureus* findings are presented for comparison.

Materials and methods

Patient material. Patients living in and around Budapest are admitted to the hospital. At the beginning of the surveillance period the hospital had 1430, and at the end, 1092 beds. The hospital consists of enteral (E), non-surgical medical and paediatric (NS), and oto-rhino-laryngological, surgical, acute and chronic respiratory (S) wards. In the S wards exclusively infectious patients are treated, coming mainly from other wards of the hospital. During the 20 years, 18 735 to 22 000 patients were admitted yearly to the hospital. The proportion of adults grew slightly as compared to that of children during the observation period.

Bacteriological samples. The number of non-faecal bacteriological cultures (NFBC) varied yearly between 15 891 and 21 294, the average being 18 030. Faecal samples sent to the laboratory almost exclusively from patients treated in gastroenterological wards were not evaluated in this paper. The results of the S and NS wards were separated. The positive *P. aeruginosa*, *Proteus*, *Klebsiella*, *E. coli* and *S. aureus* samples were registered monthly/yearly and expressed as the percentage of the cultures originating monthly/yearly from the S and NS wards. Owing to some technical errors made in the registration according to departments in 1975, the results of this year had to be evaluated as a whole without grouping. The ratio of the different samples in the NFBC (skin, ear, nose, throat, trachea, urine, bile, pus, cerebro-spinal fluid, haemoculture) did not change significantly during the examination period, so it was not necessary to evaluate them separately.

Bacteriological cultures were considered positive only if *P. aeruginosa*, *Klebsiella*, *Proteus* or *E. coli* probably had a pathological importance, i.e. when the culture contained an unusually high proportion of some of the four species, or if they were isolated from normally sterile samples, such as CSF or urine. In the latter case, the possibility of an artificial contamination was always considered. On the contrary, the laboratory judged the culture *S. aureus* positive even if only a few colonies had developed. Since patients of the chronic respiratory wards were permanently carrying their GNFP, positive results were only registered once in a month.

Bacteriological sampling, isolation and identification of the bacteria were performed with methods accepted internationally at the given time (13-16).

The case records of three groups of randomly selected patients who were *Pseudomonas*, *Klebsiella* or *Proteus* positive and were treated in NS wards were studied in 1958-59. Each group consisted of 100 persons. In the case *P. aeruginosa*, owing to the low number of positive results, the case records of 1960 were also studied. On the basis of the data, attempts were made to determine, whether the patients were only carriers or had developed a disease after the infection, and whether the colonization or infection occurred in the ward or outside the hospital. A patient displaying local and general clinical symptoms accompanied by deviations in laboratory parameters and a positive bacteriological result was regarded to be affected by GNFP. In the case of a positive bacteriological culture, but without any clinical and laboratory signs the patient was considered to have been colonized.

Intrahospital (IH) origin was presumed when a negative bacteriological finding was followed by a positive one both originating from the same site at least four days after admission. As the first bacteriological samples from the nose, throat, ear and skin could have contained a few colonies of GNFP without being regarded as positive, in these patients a shift toward a high proportion in the normal flora (dysbacteriosis) revealed IH effects. In a few cases these two possibilities could not be separated; however, this did not alter finally the epidemiological conclusions, it merely diminished the number of extrahospital (EH) colonizations and augmented the number of IH ones. Thus, the term colonization was used only for the sake of simplicity.

EH origin was decided only in the case of a positive bacteriological result obtained within the first 24 h after admission, and when the colonization or infection could not be brought into connection with the hospitalization or ambulatory examinations. E.g. the significant bacteriuria of a patient admitted from his home was considered as being IH if one year

previously he was subjected to instrumental intervention in the urological ward of an other hospital. Accordingly, though the principles of evaluation were similar to those of other authors [8, 17, 18], the decision was made individually.

All these caused difficulty in deciding the IH or EH origin of GNFP colonization or infection in some patients. These were grouped as "undefinable". In $1/3-1/4$ part of the case records the question of whether the GNFP were colonizing only transiently or caused disease could not be determined either. These were presented as "not evaluable".

The above studies were repeated in 1965-66 and 1970-71, thus a total of 900 case records was examined. This was not performed again in the successive years as a random selection of the case records could not be ensured.

Mathematical evaluation. The points in the figures given represent the logarithm of the ratio of positive cultures per year. To eliminate the optical effect of disorder due to the dispersion of data, the least square fourth degree polynomials are also shown. The curves are determined by the equation

$$y[\%] = \exp(a_0 + T(a_1 + T(a_2 + T(a_3 + Ta_4))))).$$

where $T = \tau - 1950$, and τ is the date of year.

The fourth degree polynomials showed time-intervals characterized by unidirectional and approximately uniform changes. The linear regression of the data in these intervals with the correlation coefficient is also presented in the figures. The equation of these lines is,

$$y[\%] = \exp(m(\tau_{\text{year}} - 1950) + b)$$

For statistical evaluation of the case records, the χ^2 test was used.

Results

Rate of positivity in the total of the NFBC (Fig. 1). The rate of *P. aeruginosa*, *Proteus* and *Klebsiella* was uniformly low in 1958 (1.2%, 1.1% and 1.4%, respectively). The percentage of *Pseudomonas* and *Proteus* positive cultures changed parallel during the 20 years. Both rose steeply until 1967 (5.9 and 3.9%), subsequently they decreased moderately and rose again in the last 2-3 years. In 1977 *Pseudomonas* and *Proteus* occurred at the same level (4.5 and 4.5%).

The percentage of *Klebsiella* positivity showed a similar course but reached its maximum (5.3%) as early as in 1965. This was followed by an expressed decrease, only to increase again from 1974 and finally to surpass somewhat (5.0%) the frequency of *P. aeruginosa* and *Proteus* in 1977 just like earlier in 1958.

E. coli in 1958 started from a higher level (2.2%). It rose steeply until 1963 (7.8%). Staying at this level for a few years a moderate rise occurred again until 1975 (12.5%) while in the last 2 years a slight decrease took place (1977: 9.6%) as compared to the other GNFP.

The positivity rate of *S. aureus* was very high in 1958 (8.0%). It surpassed the total rate of the four GNFP. The incidence rose further until 1961 (9.7%) displaying after this time a continuous decrease. In 1977 the rate (4.4%) was lower than that of any of the four GNFP. Thus the occurrence of *P. aeruginosa* and *Proteus* cultures showed a 4-fold, of *Klebsiella* a 3.5-fold and of *E. coli* a 4.5-fold rise, whereas the rate of *S. aureus* decreased to its half.

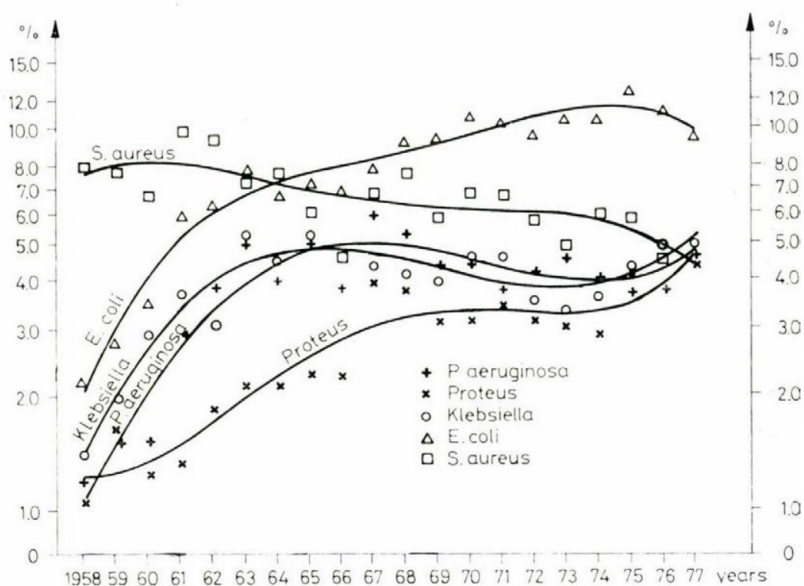


Fig. 1. Percentage of *P. aeruginosa*, *Proteus*, *Klebsiella*, *E. coli* and *S. aureus* in non-faecal bacteriological samples, 1958 to 1977. Logarithmic scale

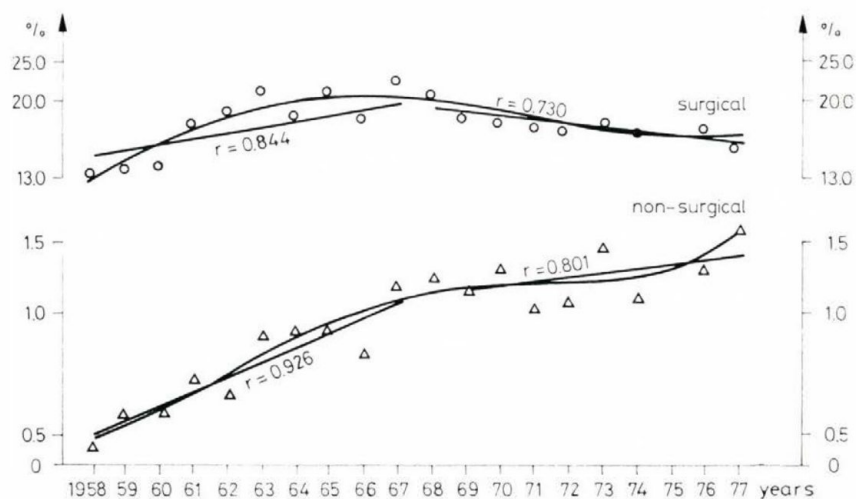


Fig. 2. Occurrence of *P. aeruginosa* in non-faecal bacteriological samples from non-surgical and surgical patients, 1958 to 1977. Without the 1975 data. Logarithmic scale

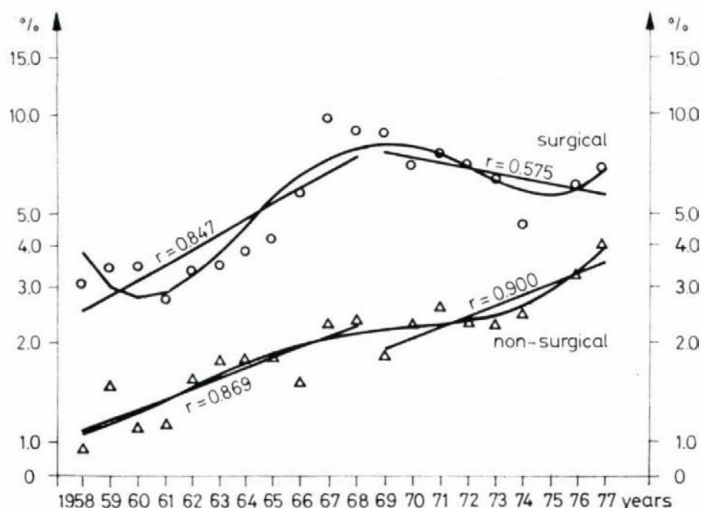


Fig. 3. Occurrence of *Proteus* in non-faecal bacteriological samples from non-surgical and surgical patients, 1958 to 1977. Without the 1975 data. Logarithmic scale

Incidence of P. aeruginosa in the NFBC from S and NS wards (Fig. 2). There was a significant difference in the positivity rate between the S and NS wards. The occurrence of *Pseudomonas* was very high in the cultures from the S wards as early as 1958 (13.3%). The rate rose further until 1967 (22.4%!), then it decreased slowly but constantly (1977: 15.3%). In contrast, in the NS ward samples the rate of *P. aeruginosa* positive cultures was very low in 1958 (0.5%). Then it started rising first rapidly until 1967 and later slower but continuously (1977: 1.6%).

Incidence of Proteus in the NFBC from S and NS wards (Fig. 3). Similarly to *P. aeruginosa*, the rate of *Proteus* positivity showed a strong increase in the samples from S wards (from 3.1% to 9.7%) till 1967, to decrease slowly until 1977 (6.8%), whereas the positive NS ward samples displayed a continuous rise in this period (1958: 1.0%; 1977: 3.9%).

Incidence of Klebsiella in the NFBC from S and NS wards (Fig. 4). In the cultures of S wards, the rate of *Klebsiella* positive findings was high already in 1958 (4.9%). This rise continued somewhat longer (1970: 11.5%) and then similarly to *P. aeruginosa* and *Proteus* a decreasing tendency could be observed until 1977 (10.3%). In the NS ward samples a rapid initial increase (1958: 1.2%, 1963: 4.3%) was followed by a decrease from 1964 (minimum 2.1% in 1973) and by a repeated increase after 1973 which continued until the end of the examination period (1977: 3.5%).

Incidence of E. coli in the NFBC from S and NS wards (Fig. 5). The incidence of *E. coli* was already high in the S wards in 1958 (6.1%). A moderate rise until 1963 (9.2%) was followed from 1965 by a stationary phase (1976: 10.9%, 1977: 8.6%). In the samples from the NS wards the ratio of *E. coli*

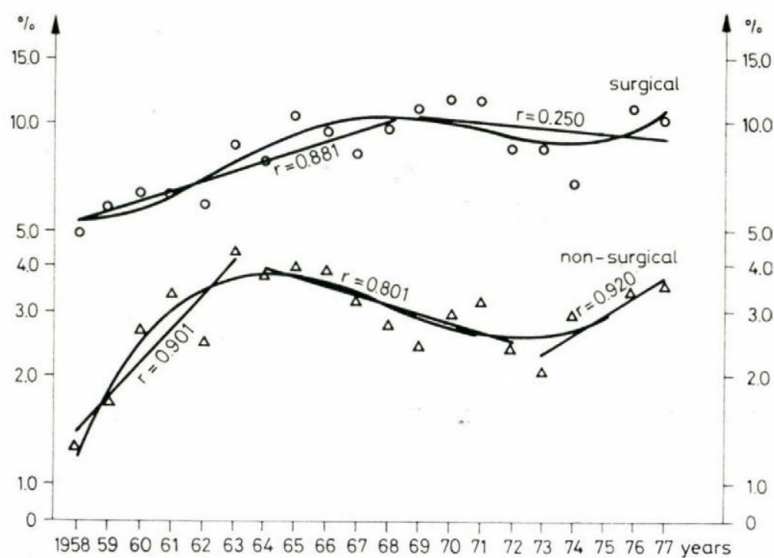


Fig. 4. Occurrence of *Klebsiella* in non-faecal bacteriological samples from non-surgical and surgical patients, 1958 to 1977. Without the 1975 data. Logarithmic scale

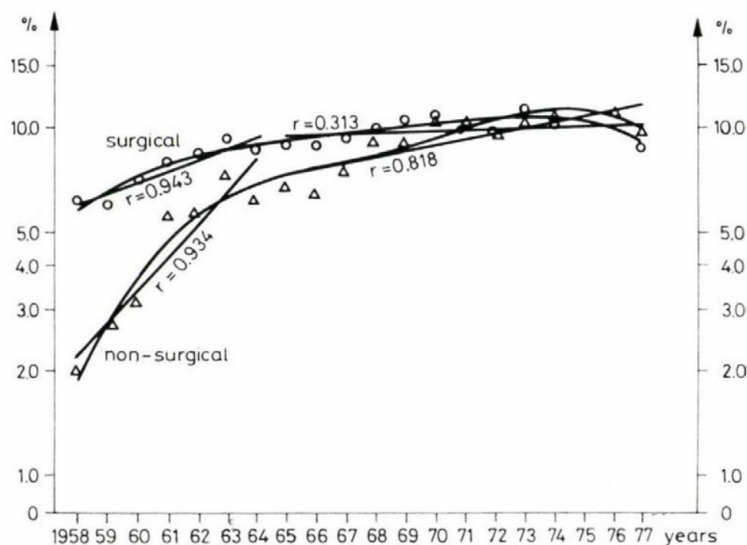


Fig. 5. Occurrence of *E. coli* in non-faecal bacteriological samples from non-surgical and surgical patients, 1958 to 1977. Without the 1975 data. Logarithmic scale

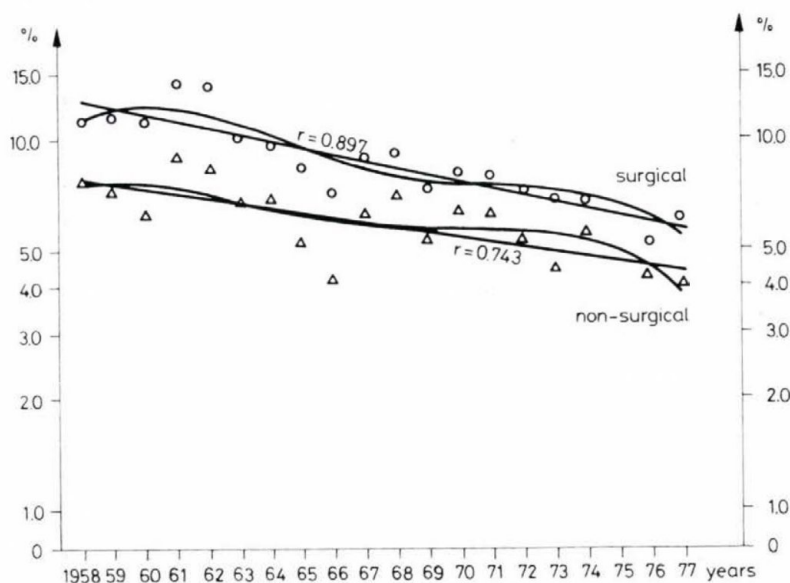


Fig. 6. Occurrence of *S. aureus* in non-faecal bacteriological samples from non-surgical and surgical patients, 1958 to 1977. Without the 1975 data. Logarithmic scale

strains predominated in 1958 (2.0%) showing a 3-fold rise until 1964 (6.2%) and a slower one (9.9%) until 1977.

Ratio of *S. aureus* in the NFBC of S and NS wards (Fig. 6). The incidence was high in both the S and NS ward samples in 1958 (11.4% and 7.8%). After a transient rise until 1961 (14.1% and 9.0%) a continuous decrease started (1977: 6.1% and 4.0%). Thus, the occurrence of *S. aureus* changed inversely to the GNFP. The decrease was similar in the S and NS wards.

IH and EH colonization and proportion of sick persons (SP) and symptomless carriers (SC). Previous studies of the S ward case records revealed hardly a few instances of EH GNFP colonization. Thus no further attempts were made in this respect in the S wards. On the contrary, the 900 case records of the NS wards showed that a significant part of GNFP colonization had taken place EH (Table I). The following average proportions of IH and EH colonizations plus infections were found: *P. aeruginosa* 4.1:1.0, *Proteus* 2.2:1.0, and *Klebsiella* 2.1:1.0 (Table II). During the examination period, these ratios underwent changes. *P. aeruginosa* rose from 3.7 to 4.4, *Proteus* from 2.1 to 2.9 and *Klebsiella* from 1.7 to 2.0. Thus, the increased occurrence of these bacteria was accompanied by a rise in the ratio of IH and EH colonization. At the same time, the proportion of SP grew as compared to SC in the case of *P. aeruginosa* from 3.2:1.0 to 5.0:1.0; in that of *Proteus* from 2.2:1.0 to 3.0:1.0; and in the case of *Klebsiella* from 2.4:1.0 to 3.2:1.0 in the period 1958–

Table I

Intrahospital and extrahospital colonization in sick patients and symptomless carriers on the basis of the case records of each 300 *Klebsiella*, *Proteus* and *P. aeruginosa* positive patients

Year of hospitalization	Colonization	Klebsiella					Proteus					P. aeruginosa				
		positivity (no. of persons)														
		Sick patient	Symptomless carrier	Sick and carrier	Not evaluable	Total	Sick patient	Symptomless carrier	Sick and carrier	Not evaluable	Total	Sick patient	Symptomless carrier	Sick and carrier	Not evaluable	Total
1958, 1959 and 1960*	Extrahospital	10	7	17	—	17	8	5	13	—	13	8	2	10	—	10
	Intrahospital	22	7	29	—	29	20	7	27	—	27	29	8	37	—	37
	Undefinable	20	8	28	26	54	21	10	31	29	60	21	8	29	24	53
	Total	52	22	74	26	100	49	22	71	29	100	58	18	76	24	100
1965 and 1966	Extrahospital	5	6	11	—	11	7	6	13	—	13	7	2	9	—	9
	Intrahospital	26	5	31	—	31	18	5	23	—	23	31	6	37	—	37
	Undefinable	26	6	32	26	58	26	7	33	31	64	17	6	23	31	54
	Total	57	17	74	26	100	51	18	69	31	100	55	14	69	31	100
1970 and 1971	Extrahospital	9	6	15	—	15	7	4	11	—	11	8	3	11	—	11
	Intrahospital	25	5	30	—	30	26	6	32	—	32	41	7	48	—	48
	Undefinable	24	7	31	24	55	24	9	33	24	57	16	3	19	22	41
	Total	58	18	76	24	100	57	19	76	24	100	65	13	78	22	100

* In 1960 only for *P. aeruginosa*

Table II

Intrahospital and extrahospital colonization in sick persons and symptomless carriers in non-surgical wards, on the basis of the case records of each 300 *Klebsiella*, *Proteus* and *P. aeruginosa* positive patients

Year of hospitalization	Klebsiella				Proteus				P. aeruginosa			
	Ratio of sick persons and symptomless carriers	Ratio of intrahospital and extrahospital colonization	Ratio sick/carrier		Ratio of sick persons and symptomless carriers	Ratio of intrahospital and extrahospital colonization	Ratio sick/carrier		Ratio of sick persons and symptomless carriers	Ratio of intrahospital and extrahospital colonization	Ratio sick/carrier	
			Intrahospital	Extrahospital			Intrahospital	Extrahospital			Intrahospital	Extrahospital
1958, 1959 and 1960*	2.36	1.71	—**	—**	2.23	2.08	—**	—**	3.22	3.70	—**	—**
1965 and 1966	3.35	2.82	—	—	2.83	1.77	—	—	3.93	4.11	—	—
1970 and 1971	3.22	2.00	—	—	3.00	2.91	—	—	5.00	4.36	—	—
1958 to 1971	2.93	2.09	4.29	1.26	2.66	2.22	3.56	1.47	3.96	4.07	4.81	3.29
			p < 0.01				p < 0.05					

* In 1960 only for *P. aeruginosa*

** Owing to the low number of cases, a common evaluation of the three examination periods had to be carried out

1971. The average values in the 300 *P. aeruginosa*, 300 *Proteus* and 300 *Klebsiella* case records were 4.0:1.0, 2.7:1.0 and 2.9:1.0, respectively.

As to the proportion of SP and SC following IH and EH colonization, the ratio of infections and symptomless carrier states was always higher after IH colonization. The proportions were in *Pseudomonas* positive cases: EH strains, SP:SC = 3.29:1.0; IH strains, SP:SC = 4.81:1.0 (Owing to the small numbers, the differences were not significant.) In *Proteus* positive cases: EH strains, SP:SC = 1.47:1.0, IH strains, SP:SC = 3.56:1.0 ($p < 0.05$); and *Klebsiella* positive cases: EH strains, SP:SC = 1.26:1.0, IH strains, SP:SC = 4.29:1.0 ($p < 0.01$).

Changes in the number of IH and EH colonizations and infections (Table III). During the examination period the number of positive cultures per patient changed only slightly, e.g. in the case of *P. aeruginosa* from 1.6 to 1.8, in that of *Proteus* from 1.4 to 1.7, and in the case of *Klebsiella* from 1.5 to 1.7. This provided means for estimation of the number of IH and EH colonizations and infections. According to the results, both IH and EH colonizations and infections showed an increase between 1958 and 1971. The number of patients who were admitted with an undoubtedly EH colonization or infection grew in the case of *P. aeruginosa* from 10 to 22, in that of *Proteus* from 32 to 43 and in the

Table III

Estimation of the number of undoubtedly intrahospital and extrahospital colonizations and infections on the basis of non-faecal bacteriological cultures and each 300 case records with P. aeruginosa, Proteus or Klebsiella positive findings

Infective agent	Year	No. of positive cultures	Average no. of positive cultures per case record	No. of positive cases	Extrahospital positive cases		Positive intrahospital cases	
					% *	estimated no.	% *	estimated no.
<i>P. aeruginosa</i>	1958, 1959 and 1960	245	1.6	153	10	10**	37	38**
	1965 and 1966	252	1.8	140	9	13	37	52
	1970 and 1971	353	1.8	196	11	22	48	94
<i>Proteus</i>	1958 and 1959	347	1.4	248	13	32	27	67
	1965 and 1966	495	1.6	309	13	40	23	71
	1970 and 1971	671	1.7	395	11	43	32	126
<i>Klebsiella</i>	1958 and 1959	418	1.5	279	17	47	29	81
	1965 and 1966	1169	1.7	688	11	76	31	213
	1970 and 1971	945	1.7	556	15	83	30	167

* See Table I. Number of extrahospital or intrahospital colonizations in 100 case records each, including sick persons and symptomless carriers

** Calculated for two years

Table IV

Changes in the number of intrahospital and extrahospital colonizations and infections in 1958-59 and 1970-71 on the basis of data presented in Table III

Infective agent	Extrahospital cases			Intrahospital cases		
	no.		ratio	no.		ratio
	1958-59	1970-71		1958-59	1970-71	
<i>P. aeruginosa</i>	10	22	2.20	38	94	2.47
<i>Proteus</i>	32	43	1.34	67	126	1.88
<i>Klebsiella</i>	47	83	1.77	81	167	2.06

case of *Klebsiella* from 47 to 83. The number of definitely IH colonizations and infections rose even more rapidly: the *Pseudomonas* positive cases from 38 to 94, the *Proteus* positive ones from 67 to 126, and the *Klebsiella* positive ones from 81 to 167. The quotient of these numbers shows more clearly the rate of increase (Table IV). Accordingly between 1958 and 1971 the rise was in EH *Pseudomonas*, *Proteus* and *Klebsiella* positive cases 2.2-, 1.3-, 1.8- and in IH cases 2.5-, 1.9- and 2.1-fold, respectively.

Discussion

The incidence of hospital infections caused by *P. aeruginosa*, *Proteus*, *Klebsiella* and *E. coli* started to increase during the staphylococcosis epidemic of the fifties [1, 4, 8, 9] and these bacteria gained ground rapidly [19-21]. The situation was similar in the László Hospital from the end of the fifties till the middle of the sixties as revealed by the present data. *Pseudomonas* and *Proteus* showed a 4-fold, *Klebsiella* a 3.5-fold and *E. coli* a 4.5-fold increase during the 20 years studied. The incidence of *S. aureus* decreased to half in the same period.

The spread of GNFP started considerably earlier in the S wards where in 1958 the percentage of *P. aeruginosa*, *E. coli*, *Klebsiella* and *Proteus* positive cultures was 13.3, 6.1, 4.9 and 3.1, respectively. The incidence reached its maximum in the second half of the sixties, decreasing then slightly till the middle of the seventies and rising again in the last years of the examination period. The decrease was probably due to new hygienic regulations, as occurred in other health institutions, too [22, 23]. This is indicated by the significantly stronger scattering of data from the end of the sixties: the correlation coefficients (r) decreased to 0.5 or even below it. The difference in the proportion of GNFP diminished by 1977, though *P. aeruginosa* kept its leading position. In 1977 the frequency in the samples of *P. aeruginosa* was 15.3%; of *Klebsiella*

10.3%; of *E. coli*, 8.6%; and of *Proteus*, 6.8%. Thus, a 20–120% rise occurred in the S wards.

The incidence of GNFP in the NS wards showed a different course. The rate of *P. aeruginosa*, *Proteus*, *Klebsiella* and *E. coli* positive cultures was 0.5, 1.0, 1.2 and 2.0%, respectively, in 1958. Accordingly, *P. aeruginosa* was present in the lowest and *E. coli* in the highest percentage and this persisted throughout the whole period studied. Similarly characteristic was the unbroken rise of *P. aeruginosa* and *Proteus* in the NS wards during the 20 year period. However, the incidence of *Klebsiella* decreased between 1963 and 1973; an explanation can hardly be found for the phenomenon. The cause might be the variation in the sensitivity of *Klebsiella* to the antibiotics used in those years, in changes in the system of disinfection, or in the increasing tolerance of *Klebsiella* to disinfectants, etc. Indeed, new hygienic regulations introduced in the respiratory wards of the hospital reduced the incidence of *Proteus* but did not control the occurrence of the more resistant *P. aeruginosa* [24]. The incidence of *E. coli* in the NS ward material grew rapidly at the beginning of the examination period, then in 1970 it reached the level of the S wards.

In 1977 the incidence of the GNFP studied in the NS wards was: *P. aeruginosa* 1.6%; *Proteus*, 3.9%; *Klebsiella*, 3.5%; and *E. coli*, 9.9%. Thus, the 3–4.5-fold rise of the occurrence of GNFP in the NS wards significantly exceeded the increase in the S wards. All these indicated that since 1960 a state of equilibrium has developed in the S wards, whereas the hygienic regulations in the NS wards were not sufficient to stabilize the situation.

The frequency of *S. aureus* decreased from 1961 parallel in the S and NS ward material which indicated the end of the staphylococcal epidemic.

The incidence of colonizations and infections of EH origin grew with all the three GNFP continuously between 1958 and 1971. The number of the IH colonizations and infections increased at an even higher rate. In the case of *Klebsiella* this growth in IH colonizations stopped in the NS wards in 1963 and a decrease occurred thereafter. Thus, the lower rate of *Klebsiella* in the NS wards was due to the lower number of IH infections in the mid-sixties. For this transient phenomenon, new antibiotics and/or new disinfectants may have been responsible.

The proportion of SP and SC followed closely these changes. Significantly higher was the rate of diseases than that of symptomless carrier states of the three GNFP after IH colonization. The deteriorated condition of the patients requiring hospitalization may have played a predisposing role, but the more expressed virulence of the IH strains cannot be ruled out either. As in the examination period the number of IH colonizations grew more rapidly than that of EH colonizations, the SP:SC proportion rose as well. When, however, the number of IH colonization of *Klebsiella* decreased in the NS wards between 1963 and 1973, the SP:SC proportion decreased immediately.

The data presented revealed not only the sudden advance of *Pseudomonas*, *Proteus* and *Klebsiella* strains in the hospital, but also the growing number of colonizations and infections occurring outside the hospital. Thus, the increasing rate of GNFP infections can be traced back not only to the causes prevailing in the hospitals. Indeed, the increasing spread of these bacteria outside the hospitals has more signs. Several papers deal with their occurrence in drinking water [25–27], in the water of swimming pools and public baths [28–30], in communal and industrial waste water [26, 31–33], in food of restaurants and hospitals [34–37], in medicaments and cosmetics [34, 36, 38, 39]. Deodorants and soaps containing disinfectants also cause a shift of the dermal microflora towards GNFP [40–42].

Healthy individuals are more, and sick persons less, resistant to repeated invasion of these bacteria [43–45] either in hospital or at home. This is the explanation for the increased number of colonizations occurring outside hospitals. Consequently, a growing number of GNFP find their way into the hospitals. Within the hospitals they have the opportunity to continue multiplying and spreading, as indicated by the increased number of nosocomial infections. Thus the advance of GNFP means not only a hospital or hygienic but also a civilisation problem.

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EXTRAHOSPITAL AND INTRAHOSPITAL FACTORS PREDISPOSING TO THE SPREAD AND COLONIZATION IN PATIENTS OF *PSEUDOMONAS* *AERUGINOSA*, *PROTEUS* AND *KLEBSIELLA* IN AN INFECTIOUS HOSPITAL

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The number of patients admitted to hospital who harbour *Pseudomonas aeruginosa*, *Proteus* and *Klebsiella*, keeps rising. Of the factors predisposing to colonization, only diabetes and antibiotic therapy exert their effect equally in extrahospital and intrahospital environment. Malignant diseases, immune suppressive therapy and instrumental interventions play a predominant role in the hospital. In extrahospital environment, infancy and old age, poor general condition as well as in almost half of the cases, an inflammatory process caused by viruses or bacteria was found to create favourable conditions for the colonization of facultative pathogens. One of the main sources of the Gram-negative facultative pathogens studied was the faeces of enteric patients in the hospital. The frequency of *P. aeruginosa*, *Klebsiella* and *Proteus* positive cultures rose parallel in the faecal and non-faecal bacteriological samples in the period 1958 to 1977. The seasonal changes observed in the frequency of positive cultures revealed that the Gram-negative facultative pathogens had increased in number first in the enteral wards, spreading subsequently to the medical and paediatric wards, and finally they appeared in a high number in the surgical wards, originating from patients transferred there from the medical or paediatric wards.

As in other parts of the world, an advance of *Pseudomonas aeruginosa*, *Klebsiella* and *Proteus* took place during the fifties and sixties in the László Central Hospital for Infectious Diseases, Budapest, while the ratio of *Staphylococcus aureus* positive cultures decreased to half in the non-faecal bacteriological cultures (NFBC) between 1958 and 1977 [1]. On the basis of 404 case records evaluable in all respects for 300 *Klebsiella*, 300 *Proteus* and 300 *P. aeruginosa* positive patients treated in medical and paediatric (non-surgical: NS) wards in 1958–59, 1965–66 and 1970–71, it was possible to state the ratio of intrahospital (IH) and extrahospital (EH) colonization of these bacteria. The 404 case records provided means to study the factors promoting EH and IH colonization. The seasonal occurrence of the three Gram-negative facultative pathogens (GNFP) studied in the faecal samples (FS) of the gastroenterological (E) as well as in the NFBC of the NS and surgical (S) wards revealed further details on the relationship of EH and IH colonization and on the way of IH spread.

Materials and methods

Patient material. The method of working up a total of 360 000 NFBC, identification of GNFP and evaluation of the case records were described in the previous report [1].

A significant number of patients with gastrointestinal disease are treated yearly in the hospital. From time to time they reach almost the half of the patients admitted. Their faecal cultures were evaluated in 1958, 1962, 1966, 1970, 1972 and 1976; their average number was 27 087 (range, 18 916 to 37 004) per year. Bacteriological findings were considered *P. aeruginosa*, *Klebsiella* or *Proteus* positive in the case of an unusually high ratio of colonies or of a pure growth in the primary culture. *Proteus* positive faecal samples were registered only in the first two years, because later the organism was regarded as a constant member of the normal gut flora. Strains cultured yearly/monthly are given in the percentage of the total cultures per year or month.

Statistical analysis was done by the χ^2 test.

Results

Antibiotic therapy preceding the colonization of GNFP (Table I). In 70–85% of the 900 cases a three-day antibiotic therapy applied within a week preceded the colonization of the bacteria. The ratio was slightly higher in

Table I

Effect of a minimum 3-day antibiotic treatment on intrahospital and extrahospital colonization of Gram-negative facultative pathogens in non-surgical patients

Organism	Site of colonization	3-day antibiotic treatment before colonization					
		applied		not applied		total	
		no.	%	no.	%	no.	%
<i>P. aeruginosa</i>	EH	26	86.7	4	13.3	30	100.0
	IH	94	77.1	28	22.9	122	100.0
	Total	120	79.0	32	21.0	152	100.0
<i>Proteus</i>	EH	27	73.0	10	27.0	37	100.0
	IH	58	70.7	24	29.3	82	100.0
	Total	85	71.4	34	28.6	119	100.0
<i>Klebsiella</i>	EH	35	81.4	8	18.6	43	100.0
	IH	66	73.3	24	26.7	90	100.0
	Total	101	75.9	32	24.1	133	100.0
Sum of <i>P. aeruginosa</i> , <i>Proteus</i> and <i>Klebsiella</i>	EH	88	80.0	22	20.0	110	100.0
	IH	218	74.2	76	25.8	294	100.0
	Total	306	75.7	98	24.3	404	100.0

Percentage calculation from small numbers was done only to facilitate comparison

EH = extrahospital

IH = intrahospital

Table II

Age distribution of non-surgical patients harbouring extrahospitally or intrahospitally colonized *P. aeruginosa*, *Proteus* or *Klebsiella* and of non-surgical control patients in the years 1958, 1959, 1965, 1966, 1970 and 1971

Age group, year	Control group		<i>P. aeruginosa</i> , <i>Proteus</i> and <i>Klebsiella</i> positive patients					
			EH		IH		Total	
	no.	%	no.	%	no.	%	no.	%
0	78	8.7	28 ^c	25.5	50 ^c	17.0	78 ^c	19.3
1-4	213	23.6	25	22.7	63	21.4	88	21.8
5-9	141	15.7	6 ^b	5.5	24 ^b	8.2	30 ^c	7.4
10-19	108	12.0	—	—	12 ^c	4.1	12 ^c	3.0
20-29	69	7.7	2 ^a	1.8	6 ^c	2.0	8 ^c	2.0
30-39	63	7.0	—	—	10 ^a	3.4	10 ^c	2.5
40-49	48	5.3	3	2.7	19	6.5	22	5.5
50-59	87	9.7	8	7.3	32	10.9	40	9.9
60-69	54	6.0	21 ^c	19.1	45 ^c	15.3	66 ^c	16.3
≥70	39	4.3	17 ^c	15.5	33 ^c	11.2	50 ^c	12.4
Total	900	100.0	110	100.0	294	100.0	404	1.000

EH = extrahospital

IH = intrahospital

^{a, b, c} significant difference as compared to the control: a = $p < 0.05$; b = $p < 0.01$; c = $p < 0.001$.

EH cases with all the three bacteria studied, though the difference was not significant even if calculating with the sum of the three species.

Predisposing role of age. The control group in Table II shows the age distribution of 300 patients selected at random in 1958-59, 1965-66 and 1970-71. The ratio of GNFP positive patients shows a marked difference in almost each age group. The frequency was significantly higher than in the controls in the age group 0 and over 60 years ($p < 0.001$), and significantly lower between 5 and 39 years ($p < 0.001$ for each group). No essential difference was found between IH and EH cases, though the ratio in the age groups 0 and over 60 was higher when the colonization occurred in the hospital.

Other predisposing factors (Table III). The majority of predisposing factors playing a role in colonization or infection due to GNFP is well known. In the present material, cachexia was found at a significant higher ratio in the case of EH colonization ($p < 0.01$) whereas malignant diseases were connected more frequently with IH colonization. Diabetes appeared to be predisposing equally in IH and EH environment. Immunosuppressive treatment or instrumental intervention preceded only IH colonization. Finally, a high number of

Table III

Factors predisposing to intrahospital and extrahospital colonization of Gram-negative facultative pathogens in non-surgical patients

Predisposing factors		Before						Total	
		<i>P. aeruginosa</i>		<i>Proteus</i>		<i>Klebsiella</i>			
		positivity							
		no.	%	no.	%	no.	%	no.	%
Poor general condition	EH	26	86.7	29	78.4	36	83.7	91	82.7
	IH	82	67.2	49	60.0	47	53.2	178	60.5 ^{sss}
Malignant disease	EH	2	6.7	—	—	1	2.3	3	2.7
	IH	3	2.5	8	9.8	5	5.6	16	5.4
Diabetes	EH	2	6.7	4	10.8	2	4.7	8	7.3
	IH	5	4.1	12	14.6	8	8.9	25	8.5
Immunosuppressive therapy	EH	—	—	—	—	—	—	—	—
	IH	3	2.5	7	8.5	2	2.2	12	4.1
Instrumental intervention	EH	—	—	—	—	—	—	—	—
	IH	51	41.8	29	35.4	23	25.6	103	35.0
Preceding bacterial infection*	EH	7	23.3	7	18.9	10	23.3	24	21.8
	IH	17	13.9	13	15.9	12	13.3	42	14.3**
Preceding viral infection*	EH	7	23.3	8	21.6	11	25.6	26	23.6
	IH	15	12.3	12	14.6	14	15.6	41	14.0 ^s
Total number of case records studied	EH	30	100.0	37	100.0	43	100.0	110	100.0
	IH	122	100.0	82	100.0	90	100.0	294	100.0

Percentage calculation from small numbers was done only to facilitate comparison; several case records revealed more than one predisposing factors

* At the site of colonization

** Between EH and IH $p < 0.10$

EH = extrahospital

IH = intrahospital

s = $p < 0.05$

sss = $p < 0.001$, as compared to EH

bacterial or viral infections were observed at the site of a subsequent colonization. These infections facilitated mainly EH colonizations. The difference in frequency between IH and EH colonizations was significant in the case of virus infections ($p < 0.05$) and bordering significance in that of bacterial infections ($p < 0.10$).

Occurrence of GNFP in the NFBC of the NS and S wards as well as in the FS of the E wards (Figs 1, 2, 3). These figures demonstrate not only the incidence of *Klebsiella*, *Proteus* and *P. aeruginosa* in NS and S wards as known from

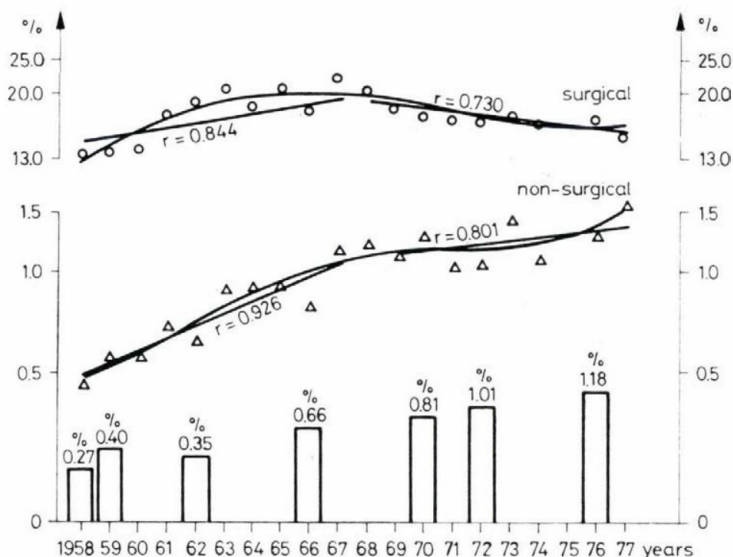


Fig. 1. Occurrence of *P. aeruginosa* in faecal and non-faecal (non-surgical and surgical) bacteriological samples, 1958 to 1977. Without the data of 1975. Logarithmic scale

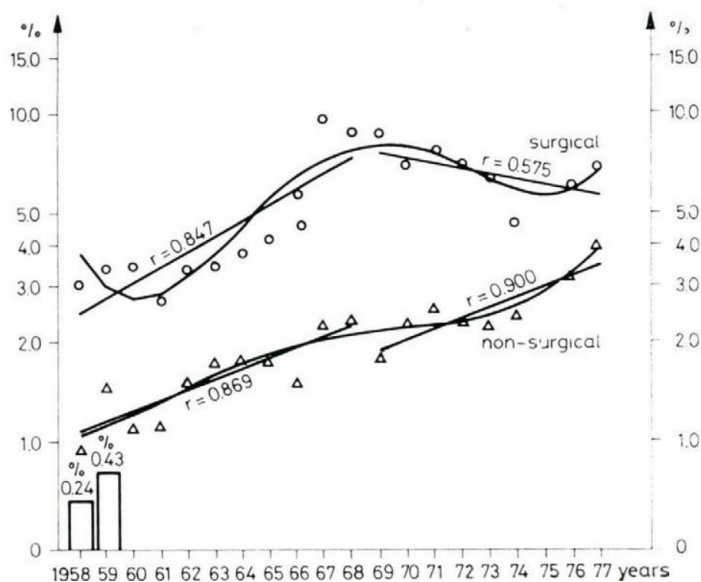


Fig. 2. Occurrence of *Proteus* in faecal and non-faecal (non-surgical and surgical) bacteriological samples, 1958 to 1977. Without the data of 1975. Logarithmic scale

the previous report [1] but also their percentage in the FS. A parallel incidence was found in the E wards and the NS wards.

Seasonal incidence of Klebsiella, Proteus and P. aeruginosa in the FS and NFBC (Figs 4, 5, 6). The peak of occurrence of *Klebsiella* in the E wards preceded by one or two months the peak in the NS wards and by somewhat

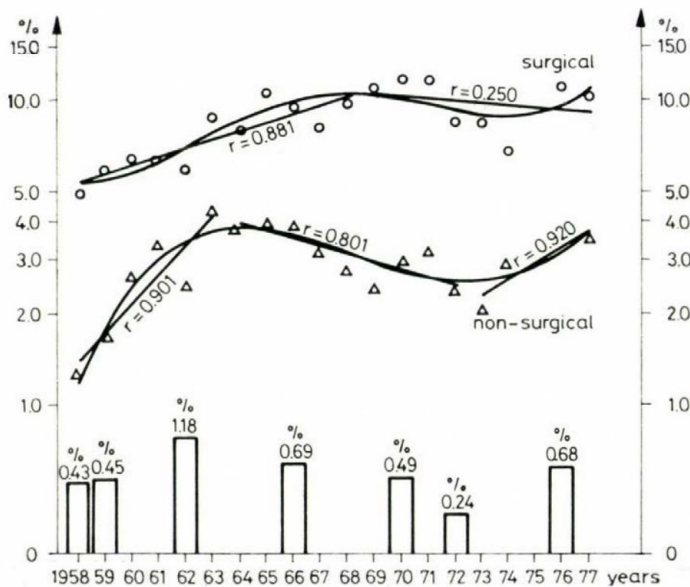


Fig. 3. Occurrence of *Klebsiella* in faecal and non-faecal (non-surgical and surgical) bacteriological samples, 1958 to 1977. Without the data of 1975. Logarithmic scale

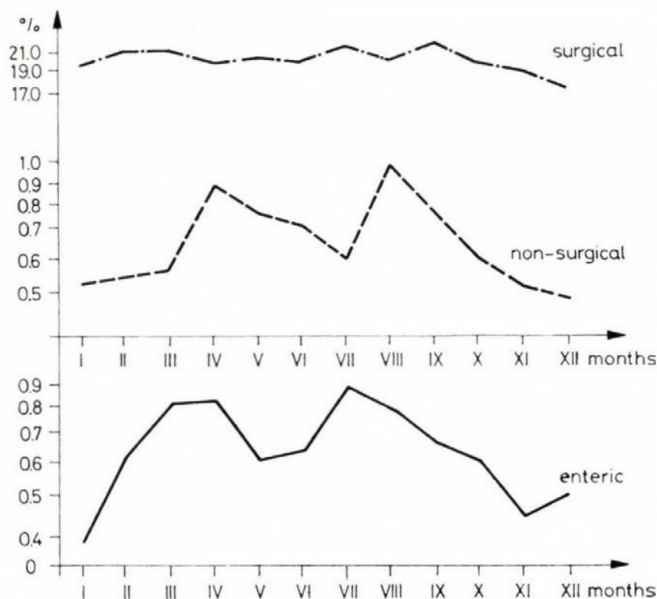


Fig. 4. Seasonal occurrence of *P. aeruginosa* in faecal and non-faecal (non-surgical and surgical) bacteriological samples. Average for the years 1958–1977. Logarithmic scale

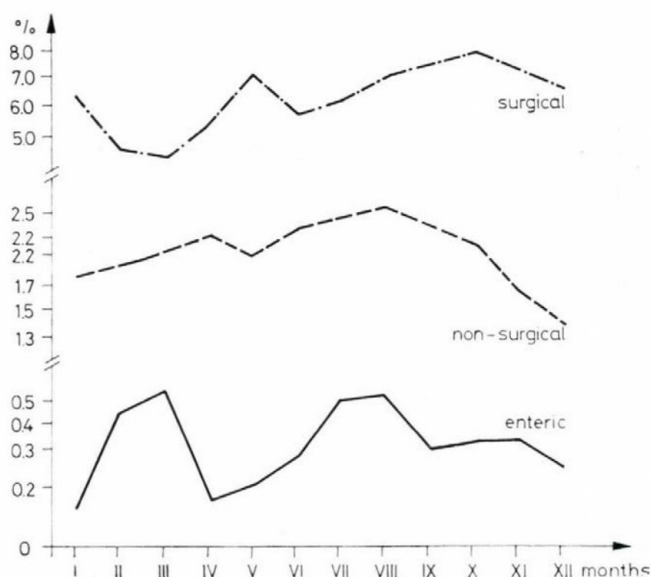


Fig. 5. Seasonal occurrence of *Proteus* in faecal and non-faecal (non-surgical and surgical) bacteriological samples. Average for the years 1958-1977. Logarithmic scale

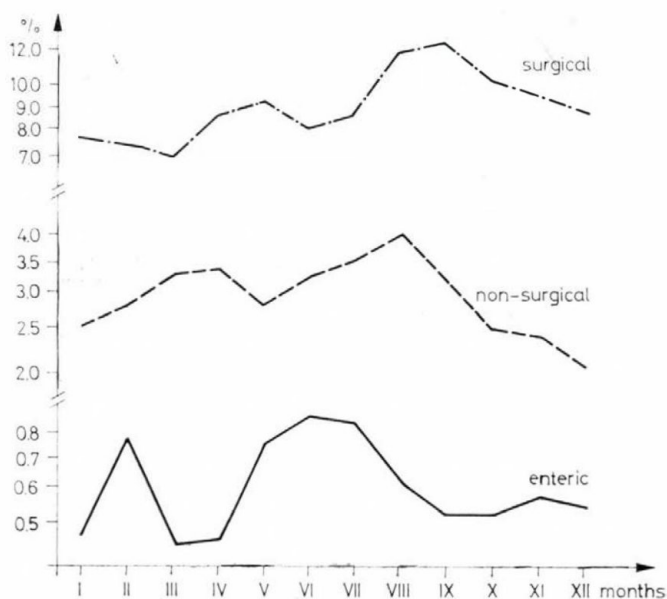


Fig. 6. Seasonal occurrence of *Klebsiella* in faecal and non-faecal (non-surgical and surgical) bacteriological samples. Average for the years 1958-1977. Logarithmic scale

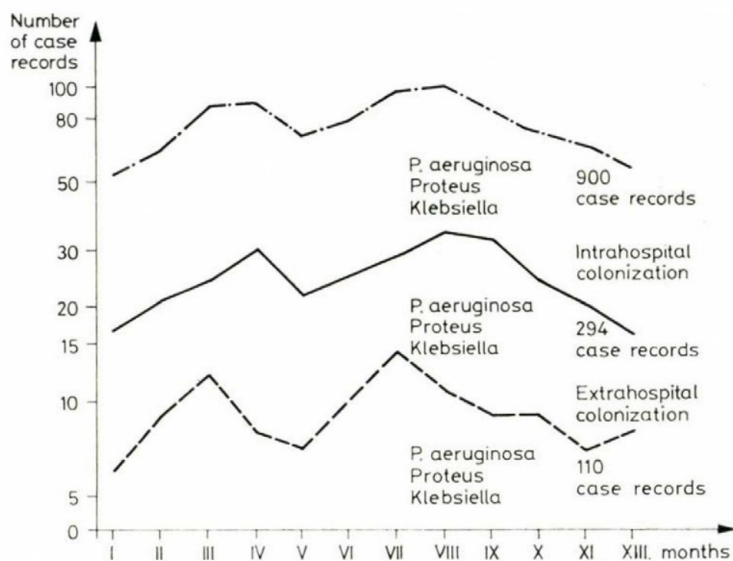


Fig. 7. Seasonal distribution of non-surgical *P. aeruginosa*, *Proteus* and *Klebsiella* positive patients on the basis of 900 case records. Logarithmic scale

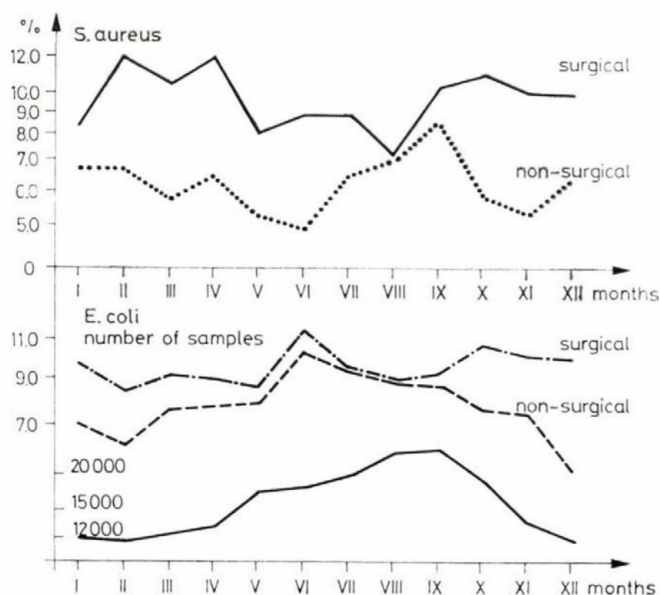


Fig. 8. Seasonal occurrence of *S. aureus* and *E. coli* in non-faecal (non-surgical and surgical) bacteriological samples, without the data of 1975, and monthly distribution of faecal samples. Average for the years 1958 to 1977

more the peak in the S wards. The amplitude of fluctuations decreased in the former sequence. *Proteus* displayed the same phenomenon. Data on *P. aeruginosa*, however, revealed its almost unchanged presence in the S wards throughout the year.

Seasonality of hospitalization of P. aeruginosa, Proteus and Klebsiella positive patients (Fig. 7). Grouping according to the date of admission the 300 *Proteus*, 300 *Klebsiella* and 300 *P. aeruginosa* positive patients, with 110 undoubtedly EH and 294 IH colonizations, the same seasonal distribution with two peaks was observed as in the E, NS and S wards. The maximum of EH cases coincided with the maximum of the positive FS of the enteric wards and the maximum of IH cases 2 months later with the maximum of the positive NFBC originating from the NS and S wards.

Seasonal changes in E. coli and S. aureus positive NFBC in the NS and S wards (Fig. 8). As compared with the other three GNFP species, the occurrence of *E. coli* in the NS and S wards remained constant during the year whereas the changes in the frequency of *S. aureus* in the S wards followed with a delay of 1-2 months the fluctuation observed in the NS wards.

Discussion

Colonization of a microorganism and its gaining predominance in the microflora is the first step of infection [2-6]. Many of the factors facilitating this process in hospitalized patients are well known [7-12]. On the other hand, conditions promoting colonization in EH environment are still not sufficiently known. The data presented refer to the altered role of the predisposing factors outside the hospital. Only antibiotic therapy and diabetes were found to exert an identical effect in both EH and IH environment. Infancy and old age as well as cachexia are more important factors outside the hospital, whereas by the time when in a malignant disease the immune resistance diminishes the patient is generally hospitalized. Immunosuppressive therapy and instrumental interventions affect mainly hospitalized patients. Surprising was the observation that at the site of a subsequent colonization, microbial inflammatory process occurred in 45% of the cases outside the hospital and in 28% in the hospital. This predisposing factor has scarcely been mentioned [13, 14]. The present results, however, indicate that this factor was the most frequently responsible for the EH colonization of GNFP.

Beside upper respiratory infections enteral infections are the most common. As *Klebsiella*, *Proteus* and *P. aeruginosa* are found in the greatest number in the intestinal tract [15, 16], enteritis may cause their most intensive spread. A significant part of the admitted patients suffered from enteral diseases. Thus it appeared reasonable to determine the incidence of *Klebsiella*, *Proteus* and *P. aeruginosa* in the FS of these patients. As expected, their inci-

dence in the enteral samples rose parallel with their frequency in the NS wards during the twenty years. A study of seasonality seemed to be the most suitable means of investigation of a relationship concerning the spread of GNFP from the E to the NS wards. Only a few authors mention a spring, end of summer or winter seasonality of infections due to GNFP [17-19]. One of us has observed a seasonality in the incidence of *Klebsiella* [20]. In the present material the incidence of the three GNFP showed a peak early in spring and a higher one in summer. The peak in the NS wards as compared to the E wards, and the peak in the S wards as compared to the NS wards, appeared with a delay of 1-2 months and the amplitude of the peaks decreased in the same order. Moreover, in this period there appeared no seasonality of *P. aeruginosa* at all in the S wards.

Grouping the *Klebsiella*, *Proteus* and *P. aeruginosa* positive cases of the NS wards according to the date of admission revealed in the case of IH colonization the same seasonal distribution as it could be observed in the positive NFBC of the NS wards. Whereas the seasonal distribution of patients who had brought the GNFP from outside the hospital corresponded to the seasonality of the positive FS.

These data as well as the fact that a significant and increasing number of patients brings along *Klebsiella*, *Proteus* and *P. aeruginosa* from EH environment when admitted to hospital [1] refers to that the supply of GNFP in the László Hospital comes from outside. The hospital environment facilitates their multiplication and one or two months later this results in colonizations and infections in the NS wards. As patients of the S wards are transferred generally from other wards of the hospital, the spread of the GNFP culminates here with a further delay. This phenomenon, however, is observable only until one of the facultative pathogens has formed a nosocomial flora in the S wards. Thereafter external factors lose their influence. This will be the explanation for the absence of seasonal fluctuations in the incidence of *P. aeruginosa* in the S wards. Indeed, earlier *P. aeruginosa* was found to have formed a nosocomial flora in the intensive respiratory ward [21].

The occurrence of *E. coli* changed parallel in the NS and S wards. The fact that everybody carries and discharges *E. coli* explains the phenomenon and this might be responsible for its different epidemiology in the hospital as compared with other GNFP. The seasonal changes in the NFBC point to the role of some still unknown factor(s) influencing its spread in the hospital.

The incidence of *S. aureus* in the NS and S wards displayed the same delay as the GNFP. The relationship again refers to that the direction of spread follows generally the shift of the patients toward the S wards in the László Hospital.

The seasonal curves of the three GNFP studied show an expressed spring peak besides the summer peak even in the positive FS. Attempts were

made to find some correlation of the spring peak with the seasonal upper respiratory diseases. The frequency of *Klebsiella*, *Proteus* and *P. aeruginosa* in the nasal and pharyngeal samples failed, however, to show such an expressed rise. Thus the high incidence in early spring requires further investigation.

The data presented reflect the special patient material and conditions of the László Hospital. Certain differences might certainly be found in other institutions, but the basic epidemiological motives are supposed to be common everywhere.

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SUCKLING MOUSE MODEL OF URINARY TRACT INFECTIONS CAUSED BY *ESCHERICHIA COLI*

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The role in the vesico-renal pathogenicity of *Escherichia coli* of mannose resistant fimbriae provisionally designated "119" has been studied in a suckling mouse model. Two to 3 days old mice were infected by route of the urinary bladder and the occurrence and character of the developing infection were investigated. (i) The fimbriated strain caused infection and inflammation of the bladder in almost 100%. In about 60% of the cases it lasted till the end of observations, the 35–37th day following inoculation. The washed, vortexed urinary bladder taken up in 5 ml of saline contained a high number (10^6 – 10^9 /ml) of bacteria, the urine was often cloudy, containing numerous leucocytes and epithelial cells packed with bacteria. The kidneys were soon involved and by the 23th to 37th day gross changes were seen in about 27%. In 10%, a classical contracted kidney developed. (ii) The nonfimbriated derivative also caused acute or chronic urinary tract infection, but with a lower frequency and the number of bacteria was also lower by 3–4 log₁₀ exponents. The model seems to be promising for the study of bacterial infections of the urinary tract and perhaps also for elucidation of its immunological and therapeutic aspects.

In previous papers [1, 2] a new mannose resistant haemagglutinin of human red blood cells has been described on *Escherichia coli* cells isolated from human urine. The factor, provisionally designated "119" fimbriae, occurred with a frequency of about 40%, and nearly 50% among haemolytic *E. coli* strains. The factor has a K88-like electron microscopic morphology, it is heat labile, relatively stable during agar-passages, but does not manifest itself under 18 °C. It is antigenically different from the known mannose resistant fimbriae K88, K99, 987P, CFI and CFII. Its production can effectively be cured; this suggests its plasmid origin. The adhesive character of 119 was observed on uroepithelial cells isolated from human urine. The adhesion of 119+ *E. coli* to the small bowel of newborn rabbits has been shown recently [3].

The characters of 119 show some similarities to the mannose resistant fimbria F8 described by ØRSKOV *et al.* [4]. The connection between 119 and F8 is under investigation.

In this paper a study of the pathological role of 119 in the suckling mouse model is presented.

Materials and methods

Strains of *E. coli* O18a,c: K5-: H-: 119+ (No. 119) and its ethidium bromide-mitomycin C cured 119- derivative (No. 119/1) were used. Both strains were alpha-haemolysin producers. A non-haemolytic mutant (designated: Kreka/7) of *E. coli* O4: K12: H5: 119+ was also used. None of the strains were ColV producers.

Animals. Conventionally bred CFLP mice (LATI, Gödöllő) were used.

Method of infection. Adult mice were injected with a volume of 0.05 ml into the tail vein.

Suckling mice at the age of 3–5 days were infected by their urinary bladder. The technical success depended chiefly on the fullness of the bladder, as at this age a bladder full of urine is clearly visible through the skin. Infection was carried out with aliquots of 0.025 ml of dilutions of fresh broth culture stained with 0.05% (final concentration) Pontamine Sky Blue (6XB, Searle, England) using a special fine cannula No. 22. It was introduced just above the symphysis, almost vertically. The stain which has no antibacterial effect, clearly indicated the success of inoculation; in this case a compact blue spot was visible above the symphysis, while an unsuccessful inoculation was indicated by a rapid spreading of the stain. In all cases the inoculum was investigated for its germ count. The infected animals were kept in their litter during the course of the experiment.

Pathological changes and bacteriological control of the infection. Experience gained in preliminary experiments has shown that in the early period of infection bacteraemia occurs. Because during bacteraemia the bacteriuria cannot correctly be evaluated, tail blood samples were taken repeatedly and the investigations were started 10–12 days later. In every case the heart blood was checked bacteriologically and mice having occasional bacteraemia were discarded.

Dissection of the animals was made under sterile conditions. First, the urinary bladder was removed and its content used for qualitative bacteriological tests. Thereafter the chopped bladder was put into 5 ml of cold, sterile saline, treated with a vortex-type apparatus (CSAV, 5001/H) for one minute. Accordingly to earlier experience, such treatment sufficiently destroys contacts made up by fimbriae. The shaken material was diluted and plated on LB agar without delay. In some experiments the urinary bladder was smeared on a slide and the Giemsa-stained cells and bacteria were studied microscopically.

Next, the kidneys were removed, inspected and weighed separately. Then they were cut open on the pelvic side and inoculated on agar plates for qualitative bacteriology, or ground in 5 ml cold, sterile saline for determination of the number of colony forming bacteria.

Finally, blood was taken by a heart puncture, and plated.

After incubation the strains of 119⁺ bacteria were controlled by 119-factor serum, while the strain 119⁻ (K⁻) by O-serum, using the slide agglutination method.

Results

Adult mice. Groups of adult mice were infected intravenously by different doses of *E. coli* No. 119 and No. 119/1 strains. Urinary tract infection was produced in about 30%, only by sublethal doses. The lasting, sometimes intermittent, and frequently lethal bacteraemia, and the low reproducibility of results was rather disturbing. Therefore, this kind of experiment was given up.

Suckling mice. Based on the observed rate of bacteriuria, inoculation of about 10⁵ germs seemed to be optimal. After such a dose, bacteraemia never occurred later than 10–12 days.

Numerous experiments were carried out with parallel groups of animals infected with fimbriated No. 119 and non-fimbriated No. 119/1 strains. Table I presents data for 60–70 mice in each group; of these 15–20 animals were killed, dissected and investigated bacteriologically on the 16th, 23rd, 31st, and 37th day following the inoculation. In all animals the absence of bacteraemia was checked.

The mice inoculated with the fimbriated No. 119 strain displayed bacteriuria which declined slowly from the early 100% rate to a frequency of

Table I

Characterization of vesico-renal infection in mice inoculated into the urinary bladder with *E. coli* 119⁺ fimbriated (No. 119) and non fimbriated (No. 119/1) forms

Character of the infection	Days following inoculation							
	16-17th		23rd		31st		37th	
	No. 119	No. 119/1	No. 119	No. 119/1	No. 119	No. 119/1	No. 119	No. 119/1
Percentage of bacteriuria	100	66	75	50	83	63	60	50**
Germ count per ml in the urinary bladder*								
mean ($\bar{x}$)	1.8×10^9	2.8×10^5	3.0×10^9	4.2×10^5	2.9×10^9	4.3×10^9	2.0×10^7	6.7×10^4
range	10^7-10^9	10^3-10^6	10^6-10^{10}	10^5-10^6	10^7-10^{10}	10^4-10^7	10^6-10^{10}	10^4-10^5
Percentage of kidneys infected	66	33	65	20	50	16	10	<10

* Taken from the vortex-treated urinary bladder in 5 ml saline

** Frequent replacement (about 60%) of the isolated agent, by other *E. coli* or *P. hauseri*

Table II

Characterization of urinary tract infection developed in mice inoculated into the urinary bladder with fimbriated, haemolytic (No. 119), haemolytic (No. 119/1), and by fimbriated, non haemolytic (Kreka/7) strains of *E. coli*

Course of infection	No. 119 (119+Hly ⁺)			No. 119/1 (119-Hly ⁺)			Kreka/7 (119+Hly ⁻)		
	days			post inoculation					
	12	17	25	12	17	25	12	17	25
Germ count in urinary bladder, mean values*	2.3×10 ⁶	1.1×10 ⁷	1.8×10 ⁶	2.8×10 ⁴	5.9×10 ⁴	4.0×10 ³	4.4×10 ⁷	7.8×10 ⁶	1.1×10 ⁶
Percentage of bacteriuria	100	100	83	80	65	45**	100	100	75
Percentage of kidneys infected	37	65	60	28	30	25	40	33	33

* Taken from the void, vortex-treated urinary bladder by washing with saline

** Frequent replacement (about 60%) of the isolated agent, by other *E. coli* or *P. hauseri*

60% on the 37th day (Table I). The non-fimbriated strain caused a similar lasting urinary tract infection in somewhat less animals. The significant difference between fimbriated and non-fimbriated strains was expressed only in the number of bacteria attached to the bladder epithelium. (Quantitative analysis was performed on the washed bladder.) The difference between fimbriated and non-fimbriated bacteria was about 3-4 log₁₀ exponents and the range values did not cover each other. The seemingly looser contact by the non-fimbriated strain was expressed by the frequent spontaneous replacement of the infecting agent by other *E. coli* or *Proteus hauseri* in the late period; this substitution was observed only in this group.

In the case of excreting animals not only a carriership, but inflammation, too, developed. The urine was often cloudy, it was mostly the first jet that showed changes. In the bladder smears numerous leucocytes and many epitheloid cells packed with bacteria were found.

The kidney infection caused by the pilated strain appeared with a frequency of about 60% and showed a slowly diminishing tendency. The non-fimbriated strain caused definitely less infections (Table I).

The strain No. 119 and its derivative No. 119/1 were both alpha-haemolysin producers. This type of haemolysin is cytotoxic in nature and therefore its role in the maintenance of infection needed further studies. Therefore, in the next series of experiments beside the strains No. 119 and No. 119/1 a Hly⁻ mutant of an 119⁺ strain of *E. coli* O4: K12: H5 was also included. According to the data presented in Table II the Hly⁻ strain possessing 119-type fimbriae caused infection of the bladder and the kidneys with a similar frequency as the fimbriated, Hly⁺ No. 119 strain. Therefore the role of 119 adhesin in the process of infection seems to be critical.

In the course of these experiments the number of infected kidneys was registered. In some cases certain gross changes were apparent mostly in the

Table III

Infection and pathological changes of kidneys of mice in the early and late periods of infection produced by E. coli No. 119 inoculated into the urinary bladder at the age of 3-5 days

Kidneys	Early (12-20 days) period				Late (23-37) period			
	gross changes	bacterial infection		gross changes	bacterial infection			
		kidneys	bladder only*		kidneys	bladder only*		
Contracted	0/35	.	.	7/68	7/ 7	$5.0 \times 10^{7**}$	0/ 7	
Slightly changed in colour	0/35	.	.	12/68	12/12	$1.2 \times 10^{6**}$	0/12	
No change	35/35	14/35	$1.8 \times 10^{5**}$	20/21	49/68	$2.5 \times 10^{3**}$	12/29	

* Presence of bacteria in the washed urinary bladder

** Bacteria/ml, taken from kidney vortexed in 5 ml saline

colour of one of the kidneys; contracted kidneys were also observed. To analyse the morphological changes and their dependence on the bladder infection, the mice were dissected at 12–20 days and then at 23–37 days. The results are summarized in Table III.

These data show that gross changes of the kidneys occurred late, after 23 days. No changes were seen earlier in 35 animals in contrast with the existing kidney infection in about 40% (14/35). On the other hand, in the early period almost 60% had an infection limited to the bladder (Table III). In the late period 19 mice out of 68 showed gross changes in one of their kidneys. These were mostly alterations in colour: one of the organs was paler than the other (12 out of 68) and in about 10% (7 out of 68) a contracted kidney had developed (Fig. 1). Such kidneys were reduced in size, weighing about 30–40% of the normal kidneys of the same litter. The surface was contracted, bulging dull and pale. Often multiple or solitary yellow patches were observed at the lower pole or near the pyeloureteral border of contracted kidneys.

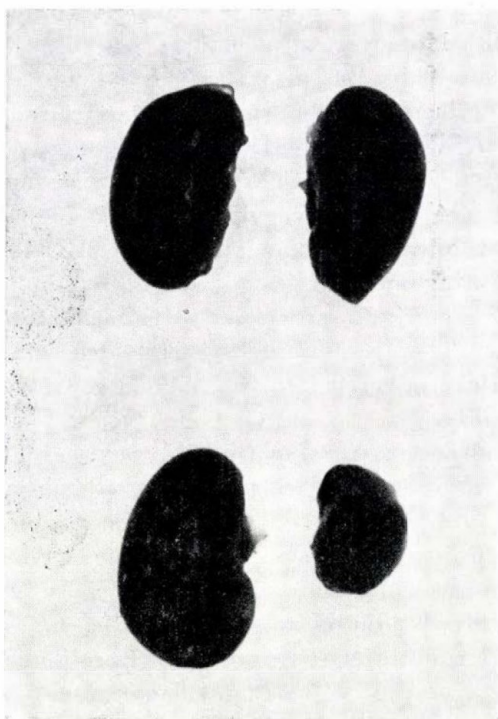


Fig. 1. Kidneys of mice at 35 days postinfection. The animals were inoculated at 3 days of age into the urinary bladder by *E. coli* O18a,c: K5: H-: 119⁺ (No. 119). Above: pair of kidneys without gross changes (control) weighing 100–110 mg each. Below: contracted kidney on right side, weighing 40 mg. The surface is contracted, bulging and dull. Patches yellow in colour on the lower pole. The other kidney is hyperaemic, compensatorily enlarged, weighing 160 mg

The other kidney was compensatorily enlarged, weighing 30–50% more than the average weight for the litter and was hyperaemic. All kidneys with suspicious morphological signs had been infected with a high number (10^6 – 10^7) of a bacteria (Table III). In this group some infected kidneys showed no changes (20 out of 49) and infection was limited to the urinary bladder in about 17%.

Deaths were infrequent and they occurred only in the early period of bacteraemia.

Discussion

To possess a reliable animal model for the study of urinary tract infections was an early aim in microbiology. The easiest way to reach this aim is perhaps by a model of haematogeneous infections [5–7]. The problem of this kind of model is that the dose required for effective infection, for instance ID_{50} , is close to the 50% lethal dose in the course of the first days [8]. Different methods were used to increase the susceptibility of animals: treatment with some chemical substances [9], massage or thermocautery of kidneys, and ureteral ligation [10]. Moreover, most urinary tract infections occur clinically by the ascending route [11]. Based on the fact that obstruction of urine flow is one of the predisposing factors in urinary tract infections, ureteral catheterization [12] and ureteral obstruction [13] were frequently applied in this kind of model. Inoculation into the urinary bladder was mostly performed by surgical procedures [14] or the inoculation was made transurethrally [15].

According to the present findings a technically simple model has been described without using any surgical or other manipulations. It allows to create symptomless bacteriuria, as well as inflammation in the lower and upper part of urinary tract. The age of animals, or in some extent the reflux caused by the volume of fluid injected into the bladder may be responsible for the observed susceptibility.

The original purpose of the experiments was to establish the role of the mannose resistant fimbriae "119" in urinary tract infections. Fimbriae (pili) confer the ability to Gram-negative bacteria to attach to epithelial cells. This in numerous instances adhesion may be one of the important factors of pathogenicity. In cases of enteric diseases, especially in enterotoxic enteropathies the first proved adhesin was the specific, mannose resistant pilus K88 [16]. In urinary tract infections caused by *E. coli* a correlation was found between the presence of type 1 (common) fimbriae and the ability to cause urinary tract infections [17]. On the other hand, ØRSKOV *et al.* [4] have recently suggested that type 1 fimbriae are adhesins for mucus and the renewing process of mucus may play a defensive function by trapping such bacteria. Other recent papers [18–21, 4] have reported on mannose resistant fimbriae in connection with urinary tract infections.

The question is not so simple whether or not a given adhesin plays a role in the infective process, because different steps of the pathogenicity may be involved starting from asymptomatic bacteriuria and cystitis, to pyelonephritis. This has already been discussed by EDÉN *et al.* [19].

Last but not least a reliable model and careful experiments are urgently needed to be able to study the antibiotic therapy and the immunological and immunopathological aspects of vesico-renal infections.

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NOTE ADDED IN PROOF

Strain No. 119 has the antigen K5 and the fimbria "119" is a complex of F8 and pseudotype 1 (personal communication by Drs F. ØRSKOV and I. ØRSKOV).

IS THE COLOUR OF THE MATURE AERIAL MYCELIUM (SPORE MASS) OF STREPTOMYCETES A DIAGNOSTIC KEY-CHARACTER OF DECISIVE TAXONOMIC IMPORTANCE?

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A white sporulated stable mutant strain (strain No. 66/m) of a *Streptomyces* sp. belonging to the *nigrescens* group of the typical grey *Streptomyces* spp. has been isolated. On the basis of ISP (International Streptomyces Project) criteria strain No. 66/m would be considered a true member of the *albus*-group of *Streptomyces* (regarded by some workers as a well separated assemblage of species) all the more so because it fits into this alien group at least in such a degree as into that from which it has originated. Caution is necessary in using the aerial mycelium colour as a distinguishing diagnostic property for establishing large intraspecific groups, series, or even in separating species of *Streptomyces*.

The colour of the well-sporulated mature aerial mycelium (spore mass) has been considered by many authors a very stable and characteristic diagnostic feature available not only for species differentiation but also for grouping and separating the species of *Streptomyces* into large intraspecific groups or series comprising morphologically and physiologically similar species [1–6]. In 1963, a system of seven colour wheels (each wheel made of colour tabs selected from the Color Harmony Manual was identified by a single colour series name) developed by TRESNER and BACKUS [7] was accepted by the Subcommittee on Taxonomy of Actinomycetes of the International Committee on Bacteriological Nomenclature [8, 9] for determining the mass colour of sporulating aerial mycelium. In Bergey's Manual [10], PRIDHAM separated the many hundred species of *Streptomyces* into seven series (White, Grey, Yellow, Red, Blue, Green and Violet) on the basis of the spore colour en masse.

Studying the actinomycete populations of various brackwater and freshwater habitats in Finland [11] we have isolated a strain of a *Streptomyces* sp. of the grey *nigrescens*-group from a culture of which a stable white sporulating mutant was selected showing therefore such a new combination of diagnostic features which is characteristic of the members of another group, the *albus*-group of *Streptomyces* considered by some workers a separate taxonomic entity within the genus. Below we present this white mutant strain and the taxonomic problems emerging on trying to identify it according to the presently suggested determining keys and taxonomic systems.

Materials and methods

On comparing the parent strain (No. 66/0) to the mutant one (No. 66/m), the descriptive criteria and standardized methods, as well as media of ISP [12], were used.

Sensitivity disks produced by the Institute for Serobacteriological Production and Research Human, Budapest, were used for detecting the susceptibility of strains to antibiotics and antimicrobial substances.

Results

Table I presents a comparison of the wild type strain, No. 66/0, with the mutant one (No. 66/m). It is seen that among the ISP diagnostic features studied only a single one the colour of the mature aerial mycelium, has changed, namely from grey ("2 fe") into white ("a"). In the grey aerial mycelium in aging cultures of the parent strain No. 66/0, an autolytic change of the hyphal filaments and spores to a black shiny mass has also been observed. In the original description of *Streptomyces (Actinomyces) nigrescens* [13], a species showing the closest similarity to strain No. 66/0, this autolytic change has been emphasized. The spore-containing mature snow-white aerial mycelium of strain No. 66/m contains long spiral sporophores, spore chains forming 1-9 turns, morphologically typical of the wild type strain No. 66/0. No symptoms of autolysis have been observed in strain No. 66/m. Electron microscopic observations did not reveal any difference between strains 66/0 and 66/m. The antibiotic sensibility spectra of the two strains were practically identical.

On studying their carbon utilization spectra, however, we found three further deficiencies of the white sporulating variant: it was unable to utilize L-fucose, lactose and sorbitol, which proved to be good carbon sources for the growth of the parent strain. The question whether there exists a correlation between the loss of ability to produce spores characteristic of the Grey series [7, 10] and the loss of capacity to utilize certain carbon compounds remains to be answered. But since these carbon compounds do not belong to the ISP diagnostic carbon sources, the lack of their utilization by strain No. 66/m cannot further change the standard ISP description of this organism.

Discussion

The grey sporulating strain No. 66/0 belongs to a group of *Streptomyces* spp. the representative species of which is *S. nigrescens* and comprises some further similar species as e.g. *S. capuensis*, *S. albofaciens*, *S. sioyaensis*, etc. These organisms are characterized by spiral sporophores, smooth spores, substrate mycelium in the yellow-brown colour series (no distinctive pigments in substrate growth) and a lack of soluble pigments including melanoids. The

Table I

Comparison of *Streptomyces* sp. strain No. 66/0 (wild type strain, *nigrescens* group) with strain No. 66/m (mutant strain, *albus* group)

	Strain No. 66/0	Strain No. 66/m		Strain No. 66/0	Strain No. 66/m
Colour series	Grey	White	Dulcitol	—	—
(spore mass)	2fe; 3fe; 2dc	a	i-Inositol	+	+
Morphology of	Spirales	Spirales	Mannitol	+	+
sporophores	1-8 turns	1-9 turns	Sorbitol	+	—
	Moisture	Moisture	α -methyl-D-glycoside	+	+
	droplets	droplets	Salicin	(—)	—
Colour of substrate	Yellow-	Yellow-	Amygdalin	—	—
mycelium	brown	brown	Sensitivity to		
Med. ISP 2	Co 5b	Co 4a	Penicillin (3 IU)	R	R
Med. ISP 3	Co 5b/c	Co 7a/b	Oxacillin (10 μ g)	R	R
Med. ISP 4	C 7b	Co 4a	Methicillin (20 μ g)	R	R
Med. ISP 5	Co 7b	Co 7b	Chloramphenicol (30 μ g)	S	HS
Colour of soluble	None	None	Oleandomycin (30 μ g)	S	S
pigment			Streptomycin — (30 μ g)	S	S
pH indicator char-	No	No	Tetracycline (30 μ g)	S	S
acter of substrate			Neomycin (100 μ g)	S	S
mycelium			Polymyxin-B (15 μ g)	(S)	(S)
Spore surface	Smooth	Smooth	Erythromycin (10 μ g)	S	S
Carbon utilization			Sulphadimidine (400 μ g)	HS	HS
Rhamnose	—	—	Nitrofurantoin (300 μ g)	(S)	S
D-Fucose	—	—	Chlortetracycline (30 μ g)	S	S
L-Fucose	+	—	Oxytetracycline (30 μ g)	S	S
Xylose	+	+	Vancomycin (50 μ g)	HS	HS
Dextrose	+	+	Kanamycin (30 μ g)	HS	HS
Mannose	+	+	Spiramycin (30 μ g)	HS	HS
Galactose	+	+	Ampicillin (20 μ g)	R	R
Fructose	+	+	Colistin (20 μ g)	R	R
Sorbose	—	—	Lincomycin (10 μ g)	(S)	(S)
Lactose	+	—	Cephalosporin (10 μ g)	R	R
Maltose	+	+	Pristinamycin (10 μ g)	R	R
Cellobiose	—	—	Nalidixic acid (30 μ g)	R	R
Sucrose	+	+	Paromomycin (50 μ g)	S	S
Melibiose	+	+	Gentamicin (20 μ g)	S	S
Melzitose	—	—	Carbenicillin (50 μ g)	R	R
Raffinose	+	+	Nystatin (100 IU)	R	R
Dextran	+	+	Co-trimoxazole (25 μ g)	HS	HS
Inulin	—	—	Clindamycin (10 μ g)	HS	HS
Glycerol	+	+			
Adonitol	+	+			

R = resistant; S = sensitive; HS = highly sensitive; (S) = slightly sensitive. The substrate mycelium colour was identified in terms of the Prause colour scale. Symbols denote, in relation to the aerial mycelium, the Tresner-Backus colour series and the number-letter code of that tab of the Tresner-Backus colour wheels which most nearly characterizes the spore mass colour

mycelium is of no pH indicator character. All of them utilize D-glucose, D-mannitol and D-fructose but they do not grow with rhamnose. The utilization of L-arabinose, D-xylose, and sucrose is variable in this group.

Among *Streptomyces* species characterized by a true white mature aerial mycelium or spore mass, there exists a small group of species including *S.*

Table II

The change of the taxonomic group position of *Streptomyces* sp. strain 66/0 owing to a single mutational event

<i>Streptomyces</i> sp. 66/0		<i>Streptomyces</i> sp. 66/m
Grey ←		→ White
	Spiral	
	Smooth	
	Yellow-brown	
	No soluble pigment	<i>S. rangoon</i>
<i>S. capuensis</i>	No pH indicator	<i>S. albus</i>
<i>S. albofaciens</i>	No melanoid pigment	<i>S. paraguayensis</i>
<i>S. nigrescens</i>	D-glucose ⁺	<i>S. cacaoi</i>
<i>S. siayaensis</i>	L-arabinose ^{var}	<i>S. almquisti</i>
	D-xylose ^{var}	
	D-mannitol ⁺	
	D-fructose ⁺	
	Rhamnose ⁻	
	Sucrose ^{var}	

rangoon, *S. albus*, *S. paraguayensis*, *S. cacaoi* and *S. almquisti*, the members of which may be characterized by the same features as given above for the species of the *nigrescens* group. The most conspicuous difference between the members of the *nigrescens*- and *albus*-groups is the colour of their aerial mass (Grey and White series, respectively). Owing to the loss of the ability to produce spores showing, en masse, the colour criteria of the Grey series of the Tresner-Backus colour wheels and becoming completely apigmented, strain No. 66/m we can place, automatically, into the *albus*-group which otherwise comprises some very similar species (Table II).

There is a high probability that many similarly deficient variants or mutants of the *nigrescens* group exist, which can be identified, after isolation and determining their features, with one of the species of the *albus*-group. Perhaps the artificially established *albus*-group and the further groups of the White *Streptomyces* series as well as every white *Streptomyces* species may be considered collections of apigmented strains of originally chromogenic *Streptomyces* species. Consequently, caution is necessary in using the spore mass colour for identification at species or intraspecific series levels. The ISP properties are not absolutely stable either. Only detailed numerical analyses based on so many properties as possible can resolve the problem of a correct taxonomic arrangement of the species of *Streptomyces*.

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DOUBLE LYSOGENY OF *MYCOBACTERIUM* *SMEGMATIS*

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While the plating efficiency of mycobacteriophage butyricum (By) on *Mycobacterium smegmatis* strain Rabinowitz (M.sm.R.) cells is less than 10^{-8} , it proved to be 5×10^{-1} on the lysogenic *Mycobacterium smegmatis* strain Rabinowitz (M.sm.R. [V72]) cells. Bacteriophage By forms plaques on M.sm.R. cells at a very low frequency; it can, however, adsorb to these cells in the same degree as to its original host. The average burst size of *Mycobacterium smegmatis* strain butyricum (M.sm.b.) cells infected with mycobacteriophage By is 60, and that of M.sm.R. (V72) superinfected with bacteriophage By is only 6. A double lysogenic strain was isolated from M.sm.R. (V72) cells surviving By phage infection.

Several types of bacteriophage-induced changes have been reported in the genus *Mycobacterium*. Thus there is an alteration from rough to smooth colony formation following lysogenization [1]. It was reported that artificially lysogenized mycobacterium strains differ in some enzymatic properties from the original strains [2–4]. Changes in antigen components due to lysogenization were also reported [5].

In this paper we report the altered phage sensitivity of *Mycobacterium smegmatis* strain Rabinowitz lysogenized by its phage V72.

Materials and methods

Bacterial and bacteriophage strains. The bacteria and mycobacteriophages used were *Mycobacterium smegmatis* strain Rabinowitz (M.sm.R.), and its phage (designated V72), and *Mycobacterium smegmatis* strain butyricum (M.sm.b.) and its phage (designated By). All the strains were obtained from Dr. EDIT VANDRA of the National Institute for Tuberculosis and Pulmonology "Korányi", Budapest.

The lysogenic *Mycobacterium smegmatis* strain Rabinowitz (M.sm.R. [V72]) was isolated in our laboratory, and it is immune to infection with V72.

Media and buffer. YRP medium containing 1.0 g NaCl, 0.7 g KH_2PO_4 , 3.0 g NH_4Cl , 2.0 g glucose, 1.0 g yeast extract (Difco), 1 ml of 1 M CaCl_2 , 1 ml of 1 M MgCl_2 , 125 ml of 0.2 M tris hydroxymethylaminomethane per litre adjusted to pH 7.2, and broth medium containing 10.0 g beef extract (Oxoid), 20 ml glycerol per litre adjusted to pH 7.2, were used for cultivating bacteria and propagating phages. Tris buffer containing 0.05 M tris hydroxymethylaminomethane and 0.004 M CaCl_2 adjusted to pH 7.4 was used to make dilutions and to store phages.

Culturing bacteria and propagation of phages. Bacterial strains were maintained on broth agar slants. In the experiments the bacteria were grown overnight at 37 °C in YRP medium aerating the Erlenmeyer flasks by shaking. Bacteriophages were propagated, concentrated, and purified as described earlier [6].

Assay of phage titres. Phage titres were assayed by the double agar layer method of ADAMS [7]. Plates of soft agar were prepared by adding 1.5 and 0.6% agar (Difco), respectively, to YRP medium.

Adsorption of bacteriophages. Bacteria grown overnight were centrifuged, resuspended in fresh medium, then the density was adjusted to an absorbancy of 0.4 at 660 nm. After incubating at 37 °C for 2 h the cells were centrifuged and suspended in tris buffer to reach again an absorbancy of 0.4 at 660 nm.

Bacteria were infected with mycobacteriophage By at a multiplicity of 1.0, and after an additional 30 min incubation at 37 °C the samples were centrifuged to remove bacteria and the adsorbed phages. The number of cells surviving phage infection and the titres of unadsorbed phages in the supernatants were determined.

One-step growth experiment. M.sm.b. and M.sm.R. (V72) cells were treated as in the adsorption experiments and infected with mycobacteriophage By at a multiplicity of 0.1. Adsorption was allowed to proceed for 30 min. The degree of adsorption was determined, the suspensions were diluted to 1:200 by adding prewarmed medium containing 0.1% Tween 80. Diluted suspensions were incubated at 37 °C and aerated by shaking. At different intervals samples were assayed for phage titre on M.sm.b. cells. Taking into consideration that the plating efficiency of phage By is independent of the host (M.sm.b., M.sm.R. [V72]) used for propagation [8], the one-step growth of phage By on M.sm.R. (V72) cells was followed only on M.sm.b. cells.

Isolation of double lysogenic mycobacterial strain. M.sm.R. (V72) cells were treated in the same way as in the adsorption experiments. Colonies formed by the cells surviving By phage infection were investigated for double lysogeny in the following way: each colony was transferred to (i) solid YRP medium, (ii) solid YRP medium covered with M.sm.b. lawn, and (iii) solid YRP medium covered with M.sm.R. lawn. Under these circumstances all the colonies lysed M.sm.R. lawn in consequence of spontaneous phage production of the M.sm.R. (V72) cells; besides, some colonies lysed the M.sm.b. lawn, too. One of these latter colonies was inoculated into YRP medium containing 0.1% Tween 80, grown, and plated.

The colonies developed were transferred again to solid YRP medium and to the same medium covered with bacterial lawns. The procedure was repeated several times.

The strain obtained was investigated for double lysogeny and also for pseudolysogeny by the method described by GRANGE and BIRD [9].

Results and discussion

Plating efficiency of mycobacteriophage By was determined with overnight grown cells of M.sm.b., M.sm.R., and M.sm.R. (V72) strains. Plating efficiency of phage By determined on M.sm.b. cells was taken as the unit. Table I shows that mycobacteriophage By gives plaques at a frequency of less than 10^{-8} on M.sm.R. cells and 5×10^{-1} on M.sm.R. (V72) cells, respectively. The plating efficiency was independent of the media used.

A difference in plaque morphology could be also observed. Plaques on M.sm.R. (V72) cells were usually smaller than those on M.sm.b. cells (Fig. 1).

To follow the time course of phage production, M.sm.b. and M.sm.R. (V72) cells were infected with phage By at a multiplicity of 0.1 as described in Materials and methods. Samples were obtained at 30 min intervals and

Table I
Plating efficiency of mycobacteriophage By on different mycobacteria

	M.sm.b.	M.sm.R.	M.sm.R. (V72)
Efficiency of plating	1	$<10^{-8}$	5×10^{-1}

assayed for infectivity on M.sm.b. cells. Using YRP medium, the latent period was equally 60 min on the two hosts, the average burst size on M.sm.b. cells was 60, while that on M.sm.R. (V72) was only 6 (Fig. 2).



Fig. 1. Plaques of phage By formed on M.sm.b. (left) and M.sm.R. (V72) (right) cells

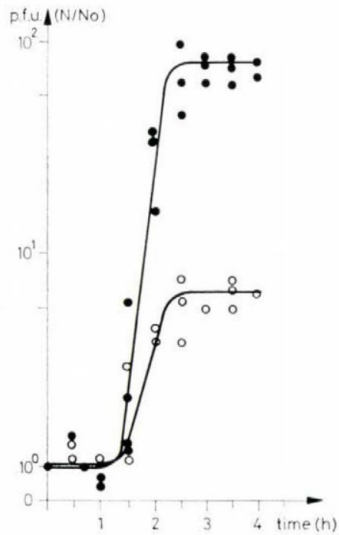


Fig. 2. Phage production of M.sm.b. (●) and M.sm.R. (V72) (○) cells infected with phage By in YRP medium

The difference in the burst size of mycobacteriophage By on different hosts is comparable to the characteristics of mycobacteriophage D29, which was found to have a different latent period, raise period, and average burst size on different hosts [10].

As alteration of phage sensitivity of *Salmonella anatum* after lysogenization is a well-known phenomenon where alteration of phage receptors and that

Table II
Adsorption of mycobacteriophage By to different mycobacteria

Bacteria	Per cent of phage adsorption measured by the titre of unadsorbed phages	Per cent of cells killed by phage infection
M.sm.b.	82	81
M.sm.R.	70	0
M.sm.R. (V72)	80	30

of antigenic composition run in parallel [11], we hypothesized that the difference between the plating efficiency of phage By on M.sm.R. and M.sm.R. V(72) cells was the consequence of the changed adsorbing ability of the lysogenized M.sm.R. cells.

To test this hypothesis we determined By phage adsorption to M.sm.b., M.sm.R., and M.sm.R. (V72) cells. The degree of phage adsorption measured by the titre of unadsorbed phages and number of cells killed by phage infection were analyzed. Table II shows that By phage adsorption to the three strains measured by the titre of unadsorbed phages proved to be similar and varied between 70 and 82%; on the contrary, the number of bacteria killed by phage infection varied from 0 to 81%. The fact that By phage equally adsorbs to M.sm.R. and its lysogenic variant means that the difference in the plating efficiency on the two strains cannot be explained by an alteration of the receptors after lysogenization. The per cent of killed cells by By phage infection shows that (i) all the phage adsorbing original host cells are killed by the phage infection; (ii) By phage adsorbs to M.sm.R. cells, but does not lyse them; and (iii) By phage adsorbs to the lysogenic M.sm.R. cells, but only part of these cells are lysed.

Colonies formed by M.sm.R. (V72) cells surviving By phage infection were investigated for double lysogeny on M.sm.b. and M.sm.R. cells. In a series of experiments 1-5% of these colonies was able to lyse not only the M.sm.R. lawn, but the M.sm.b. lawn, too. Colonies found to lyse M.sm.b. cells were serially transferred to YRP medium containing 0.1% Tween 80 and reisolated as described in Materials and methods. In this way a mycobacterial strain

producing both phage By and phage V72 was isolated. This strain was designated M.sm.R. (V72, By).

After five month, the M.sm.R. (V72, By) strain was checked for double lysogeny; it was found that 99% of its colonies produced both By and V72 phages. Spontaneous appearance of phage plaques could not be detected when the M.sm.R. (V72, By) cells were incorporated in soft agar overlays. The cells proved to be immune to superinfection with phage By and phage V72. Regarding the low level of prophage segregation and the lack of appearance of spontaneous phage plaques, M.sm.R. (V72, By) seems to be a true double lysogenic strain. In the literature we could not find data about double lysogenic mycobacterial strains.

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SELECTION OF MOUSE VIRULENT NON-MOTILE STRAINS OF *ESCHERICHIA COLI* BY THE SOFT AGAR TECHNIQUE

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Using strain SME-12 of *Escherichia coli* and its variants A and B showing large round, diffuse and compact-type colonial morphology, respectively, in soft-agar medium, their capsule formation, cell volume indices, toxicities and virulence in mice were determined. The results showed that although these strains were similar in toxicity, the parent strain exhibited a large capsule, a large cell volume and a high mouse virulence. Variant A had no capsule, its cell volume was remarkably lower than that of the original strain, and was avirulent; variant B had no capsule and displayed the lowest cell volume and mouse virulence. With 193 fresh isolates of *E. coli*, the majority of colonies of 16, 152 and 25 strains were of large round, diffuse and compact types, respectively. Fifteen strains of pure growth type from each of the three groups were tested for mouse virulence. The majority of strains showing large round-type growth was virulent, diffuse-type strains displayed a low virulence, while no mouse was killed by compact-type strains.

Relation of encapsulation to the colonial morphology of strains of *Staphylococcus aureus* [1–3], group A streptococci [4], *Klebsiella pneumoniae* [5] and *Klebsiella ozaenae* [6] in serum-soft agar or soft agar media has been reported. The encapsulated strains showed a higher mouse virulence than did the unencapsulated variants. The significance of the capsule for infection with strains of *Escherichia coli* has been raised as an important problem in recent years [7]. Attempts were, therefore, made whether the soft agar technique was suitable for the selection of virulent strains of this species of bacteria. The present paper reports on these investigations.

Materials and methods

Strains. Strain SME-12 of non-motile *E. coli* and its variants A and B were used. Their colonial morphologies were large round, diffuse and compact type in soft agar medium. Also, 193 strains of non-motile *E. coli* obtained from the Clinical Laboratory, St. Marianna University Medical School Hospital were used. Every strain was a fresh isolate, identified as *E. coli* according to the description of ØRSKOV [8] and was non-typable by specific antisera for pathogenic *E. coli* (Bacto-*E. coli* specific OB and OK antisera, Difco).

Determination of colonial morphology in soft agar medium. Colonial morphology of the strains in soft agar medium was determined by the method described elsewhere [5]; the strains cultured in brain heart infusion (BHI, Difco) broth at 37 °C for 18 h were diluted 1 to 10⁶ with sterile saline. To 0.1 ml of these cell suspensions, 10 ml of BHI broth containing agar (Bactoagar, Difco) at 0.15% final concentration were added, left to stand at 4 °C for 30 min

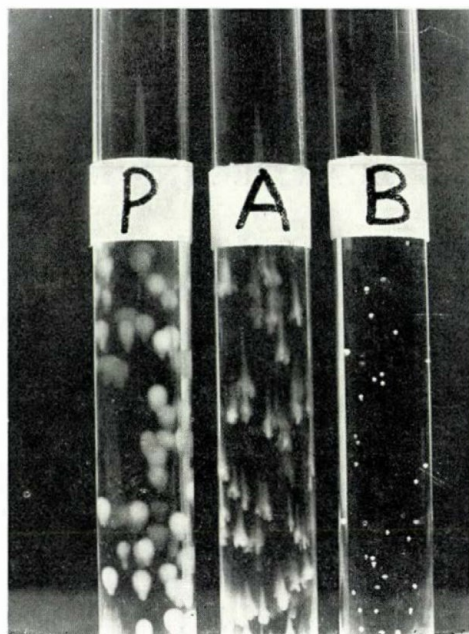


Fig. 1. Colonial morphology of strain SME-12 of *E. coli* and its variants A and B in soft agar medium cultured at 37 °C for 18 h. P, A and B mean the original strain and its variants A and B, respectively

and cultured at 37 °C for 18 h. Then, colonial morphology was determined. The types were designated as large round, diffuse and compact, as shown in Fig. 1.

Negative staining method. For determination of the capsule, the organisms cultured on BHI agar plate at 37 °C for 18 h were stained by the India ink method described by Edwards and Ewing [9] and estimated under the light microscope.

Determination of cell volume index. The cell volume index of strain SME-12 and its variants was measured by a method described elsewhere [3], by using Hopkins tubes.

Preparation and toxicity of heat-killed vaccine in mice. The organisms were cultured in BHI broth at 37 °C overnight. The bacteria were washed twice and resuspended in distilled water, autoclaved at 121 °C for 15 min, then lyophilized. They were resuspended in saline and 0.5 ml of saline containing 1, 3 and 10 mg of lyophilized organisms was injected intraperitoneally into a group of 5 mice. The number of dead animals was recorded 2 weeks after the injection.

Determination of mouse virulence. Strain SME-12 and its variants were cultured in BHI broth at 37 °C for 18 h, washed once by centrifugation with saline and cell suspensions showing nephelometrically 1.0 O. D. at 430 nm were prepared. Viable cell numbers per ml of these cell suspensions were 1.98 to 2.45×10^9 by the plate count method. They were diluted serially 10-fold with sterile saline and 0.5 ml of each dilution was injected intraperitoneally into a group of 10 mice. The DD strain of conventional mice (Nihon Clea Farm, Kawasaki, Japan) weighing approximately 18 g was used. Also, 0.5 ml of the cell suspensions was diluted 10-fold with 5% gastric mucin (Wilson type, Chicago, U.S.A.) in saline and injected into a group of 10 mice. The number of dead animals was recorded for 4 weeks after the challenge infection. For determination of mouse virulence of the other 193 strains of *E. coli*, a group of 15 pure cultures exhibiting three different colonial morphologies in soft agar, i.e. large round, diffuse, and compact type growths, were randomly selected and tentatively regarded as representatives of each growth type. These were cultured and cell suspensions were prepared as mentioned above. Then, 0.5 ml saline and 5% mucin containing 10^6 and 10^4 organisms, respectively, were prepared. These were injected intraperitoneally into groups of 5 mice. Recording of the dead mice was done as noted above. In these experiments, strains capable of killing more than 4 and less than 3 out of 5 mice were tentatively regarded as virulent and avirulent, respectively.

Results

Encapsulation of strain SME-12 and its variants. The original SME-12 strain exhibiting large round colonial morphology in soft agar medium showed a large capsule under the light microscope. No capsule was observed with the diffuse and compact-type variants appearing during subcultures of the original strain. Cell volume indices for the original strain and the diffuse and compact-type variants were 50.0, 1.05 and 0.5, respectively, indicating the existence of a large capsule in the parent strain.

Mouse virulence of strain SME-12 and its variants. On challenge with 10^6 organisms of the parent strain in saline, 100% of the animals died while 10^8 organisms of variant A exhibited a similar virulence but no mouse was killed even by 10^9 organisms of variant B. The difference in virulence between the parent strain and its variants was even more distinct on mucin challenge. On challenge with 10^3 organisms of the parent strain in 5% mucin, 100% of the mice died. Of variant A, 10^7 organisms exhibited a similar virulence, while no mouse was killed by 10^8 organisms of variant B, as shown in Table I.

Toxicity for mice of strain SME-12 and its variants. Of the parent strain SME-12 and its variants, 3 mg of a heat-killed vaccine caused death in 40 to 60% and 10 mg of any strain was 100% lethal, as shown in Table II. These results indicate that all organisms contained equal amounts of endotoxin.

Colonial morphology of 193 fresh isolates in soft agar medium. When more than 300 colonies of each strain were subjected to determination of the colonial morphology, 16 (8.3%) out of 193 fresh isolates showed mostly large round

Table I

Encapsulation, cell volume index and mouse virulence of strain SME-12 of E. coli and its variants A and B

	SME-12	Variant A	Variant B
Capsule	+	—	—
Cell volume index	50.0	1.05	0.50
Mouse virulence (challenge dose)			
10^1	2/10*	0/10	N.D.**
10^2	7/10	0/10	N.D.
10^3	10/10	0/10	N.D.
10^4	10/10	0/10	N.D.
10^5	10/10	4/10	N.D.
10^6	10/10	9/10	0/10
10^7	10/10	10/10	0/10
10^8	10/10	10/10	0/10

* Number of dead mice in groups of 10 animals

** Not done

Table II

Mouse toxicity of heat-killed vaccine prepared from parent strain SME-12 of E. coli and its variants

Heat-killed organisms (mg)	SME-12	Variant A	Variant B
10	5/5	5/5	5/5
3	3/5	3/5	2/5
1	0/5	0/5	0/5

colonies. Diffuse and/or compact colonies were usually observed in these strains, except for 3 strains which formed only large round colonies.

One hundred fifty two strains (78.7%) showed mostly diffuse-type growth including 121 strains displaying single colonial growth. Twenty-five strains (12.9%) exhibited compact-type growth including 7 strains of single colonial growth type. These results indicate that the majority of fresh isolates of *E. coli* exhibited diffuse growth.

Mouse virulence of representative strains exhibiting different colonial morphology. The experiments were designed to ascertain whether the relationship between colonial morphology of the strains and their virulence was of universal validity. Out of 15 strains for each colonial morphology group, 14 of the large round, 1 of the diffuse and no strain of the compact type growth was mouse virulent, as measured by either saline or mucin challenge. These results indicate that the majority of large round-type strains were virulent, diffuse-type strains were avirulent with few exceptions, and all compact-type strains were avirulent.

Discussion

The serum-soft agar technique has been applied for the identification of encapsulation in strains of *S. aureus* [1-3, 10]. These organisms are assumed to be exceptional in producing compact-type colonies in serum-soft agar, since their compact-colony forming active substance converts fibrinogen and fibrinogen degradation products to fibrin [11]. Recently, we have studied the relation of colonial morphology in soft agar to encapsulation and mouse virulence in strains of group A streptococci [4], *K. pneumoniae* [5] and *K. ozaenae* [6].

In these experiments, non-motile strains of *E. coli* had to be studied, since the technique applied cannot be used with motile organisms. Results with *E. coli* strain SME-12 and its variants support the data concerning the relation of colonial morphology in soft agar to encapsulation and mouse virulence.

When this technique was applied for a number of serologically non-typable strains of *E. coli* freshly isolated from human clinical specimens, 78.7% of the non-motile strains showed a diffuse-type growth, and 8.3 and 12.9% were assumed to be encapsulated and not encapsulated, respectively. This would mean that the large round-type growth of the organism was originally smooth while the compact-type growth was rough, although their colonial types on solid media could not clearly be distinguished from each other.

The 15 representative strains exhibiting large round colonies, were highly mouse virulent with few exceptions while the majority of diffuse-type strains was avirulent except for a few strains, suggesting that they would produce a small or partial capsule like the unencapsulated *S. aureus* strains of YOSHIDA *et al.* [12]. On the other hand, the compact-type organisms were certainly avirulent for mice.

The role of the K antigen in infection with strains of *E. coli* [7] has been emphasized. It may be assumed that a large amount of K antigen existed in the large round-type strains and small amounts of it in diffuse-type and compact-type strains.

NAGY *et al.* [13] and MOON *et al.* [14] postulated pili in the adhesion of the organisms to the small intestinal epithelial cells. Also, the KL capsular polysaccharide substance was noted by ROBBINS *et al.* [15] as a pathogenic factor. The soft agar technique is regarded as suitable for the selection of mouse virulent strains from non-motile strains of *E. coli*. The relation of encapsulation to the above substances should be elucidated. Further, the use of the soft agar technique needs to be investigated for the selection of virulent strains of motile *E. coli*. These problems are currently in progress in our laboratory.

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NOTE

INHIBITORY EFFECT OF AMIKACIN ON *MYCOBACTERIUM TUBERCULOSIS* IN VITRO

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(Received September 18, 1980)

Amikacin inhibited the growth of *Mycobacterium tuberculosis* strains in Šula medium at 0.1–0.2 µg/ml. As compared to the size of inoculum (approximately 10^5 – 10^6), the number of resistant mutants was low (0–9).

The antituberculous effect of the kanamycin derivative, Amikacin (BB-K8, Bristol Myers, Canada) was reported in a previous paper [1] with special regard to *Mycobacterium tuberculosis* strains resistant to other drugs. In the present study the inhibitory effect was examined as a function of live bacterial counts.

Methods. Sixteen *M. tuberculosis* cultures isolated from patients were homogenized with steel beads in physiological saline. The suspensions were adjusted turbidimetrically to the same density. A series of 10-fold dilutions of each strain was made in Sauton medium. The undiluted suspension and its 10^{-2} dilution were inoculated at 0.1 ml aliquots into a series of Šula medium containing graded doses of Amikacin (0.01, 0.02, 0.05, 0.1 and 0.2 µg/ml). For determination of the live bacterial counts, the 10^{-3} , 10^{-4} , 10^{-5} , 10^{-6} and 10^{-7} dilutions were each seeded at 0.1 ml aliquots onto three Löwenstein–Jensen slants. The media were incubated in an upright position at 37 °C and read at one week intervals. The number of live bacteria in the inoculum was estimated at the 95% confidence limits by colony-counting on Löwenstein–Jensen media with 10–60 colonies.

Results and discussion. The minimum inhibitory concentration was 0.1–0.2 µg/ml in Šula medium inoculated with the undiluted suspensions. Ten out of the 16 suspensions diluted 10^{-2} showed a lower MIC (0.1–0.05 µg/ml). Table I shows that from Šula medium containing 0.2 µg Amikacin per ml 0–9 colonies were recovered, i.e. the number of resistant cells was small as compared to the number of inoculated cells (approximately 10^5 – 10^6).

The marked inhibitory action *in vitro*, susceptibility of strains resistant to other drugs [1] and the small number of resistant mutants in the population suggest that Amikacin may be an effective antituberculous agent. The

Table I

In vitro inhibitory effect of Amikacin on *M. tuberculosis* strains after incubation for 35 days

Strain	Colony counts (95% confidence limits) of undiluted suspensions inoculated into Sula media	Colony counts in Sula medium with Amikacin (0.2 µg/ml) inoculated with undiluted suspension
A	2.8×10^6 – 1.7×10^6	3–1
B	3.8×10^5 – 2.5×10^5	0–0
C	4.9×10^6 – 3.4×10^6	2–0
D	1.8×10^6 – 9.7×10^5	1–0
E	1.8×10^6 – 9.7×10^5	0–0
F	5.9×10^6 – 4.3×10^6	0–0
G	3.7×10^6 – 2.4×10^6	2–0
H	2.7×10^6 – 1.4×10^6	9–5
I	3.4×10^5 – 2.2×10^5	0–0
J	4.2×10^6 – 2.8×10^6	1–0
K	3.1×10^6 – 1.9×10^6	7–0
L	1.8×10^5 – 1.0×10^5	5–1
M	1.1×10^6 – 3.8×10^5	0–0
N	2.5×10^6 – 1.2×10^5	0–0
O	2.7×10^5 – 1.4×10^5	0–0
P	3.5×10^5 – 2.0×10^5	1–0

results indicate that animal experiments and a subsequent clinical trial is justified, especially in view of the fact that Amikacin is less toxic than kanamycin.

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INDEX

<i>Delgado, J. M., Herrera, L. S.</i> : Protoplast Fusion in the Yeast <i>Candida utilis</i>	339
<i>Nguyen Ngoc Thuy, Galgóczy, J., Novák, E. K.</i> : Morphogenetic Effect of L-Cysteine on Dermatophytes	347
<i>Anderlik, P., Wessely, M., Szeri, I., Bános, Zs., Rápolthy, I., Budai, J.</i> : Formalin Stress Reaction of Germfree and Conventional Mice Treated with <i>Bordetella pertussis</i> Vaccine	359
<i>Petrás, Gy., Bognár, Sz.</i> : Origin and Spread of <i>Pseudomonas aeruginosa</i> , <i>Proteus</i> and <i>Klebsiella</i> during Twenty Years in an Infectious Hospital	367
<i>Petrás, Gy., Bognár, Sz.</i> : Extrahospital and Intrahospital Factors Predisposing to the Spread and Colonization in Patients of <i>Pseudomonas aeruginosa</i> , <i>Proteus</i> and <i>Klebsiella</i> in an Infectious Hospital	381
<i>Kétyi, I.</i> : Suckling Mouse Model of Urinary Tract Infections Caused by <i>Escherichia coli</i>	393
<i>Sivonen, K., Szabó, I. M.</i> : Is the Colour of the Mature Aerial Mycelium (Spore Mass) of Streptomycetes a Diagnostic Key-Character of Decisive Taxonomic Importance?	401
<i>Háber, K. J., Vajda, B. P., Földes, I.</i> : Double Lysogeny of <i>Mycobacterium smegmatis</i> ...	407
<i>Takahashi, M., Ichiman, Y., Yoshida, K.</i> : Selection of Mouse Virulent Non-Motile Strains of <i>Escherichia coli</i> by the Soft Agar Technique	413
<i>Fodor, T.</i> : Note. Inhibitory Effect of Amikacin on <i>Mycobacterium tuberculosis in vitro</i>	419
Books Received	421

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